Terapia Genica

Intervento terapeutico basato sulla modificazione del patrimonio genetico di una cellula somatica, allo scopo di correggere un difetto genetico o di fornire una nuova funzione biologica per combattere una patologia.

Gli obiettivi della terapia genica

1. Malattie genetiche: immunodeficienze, emofilia, talassemia, distrofia muscolare, genodermatosi, malattie lisosomiali, fibrosi cistica

2. Malattie metaboliche e autoimmuni: diabete, sclerosi multipla

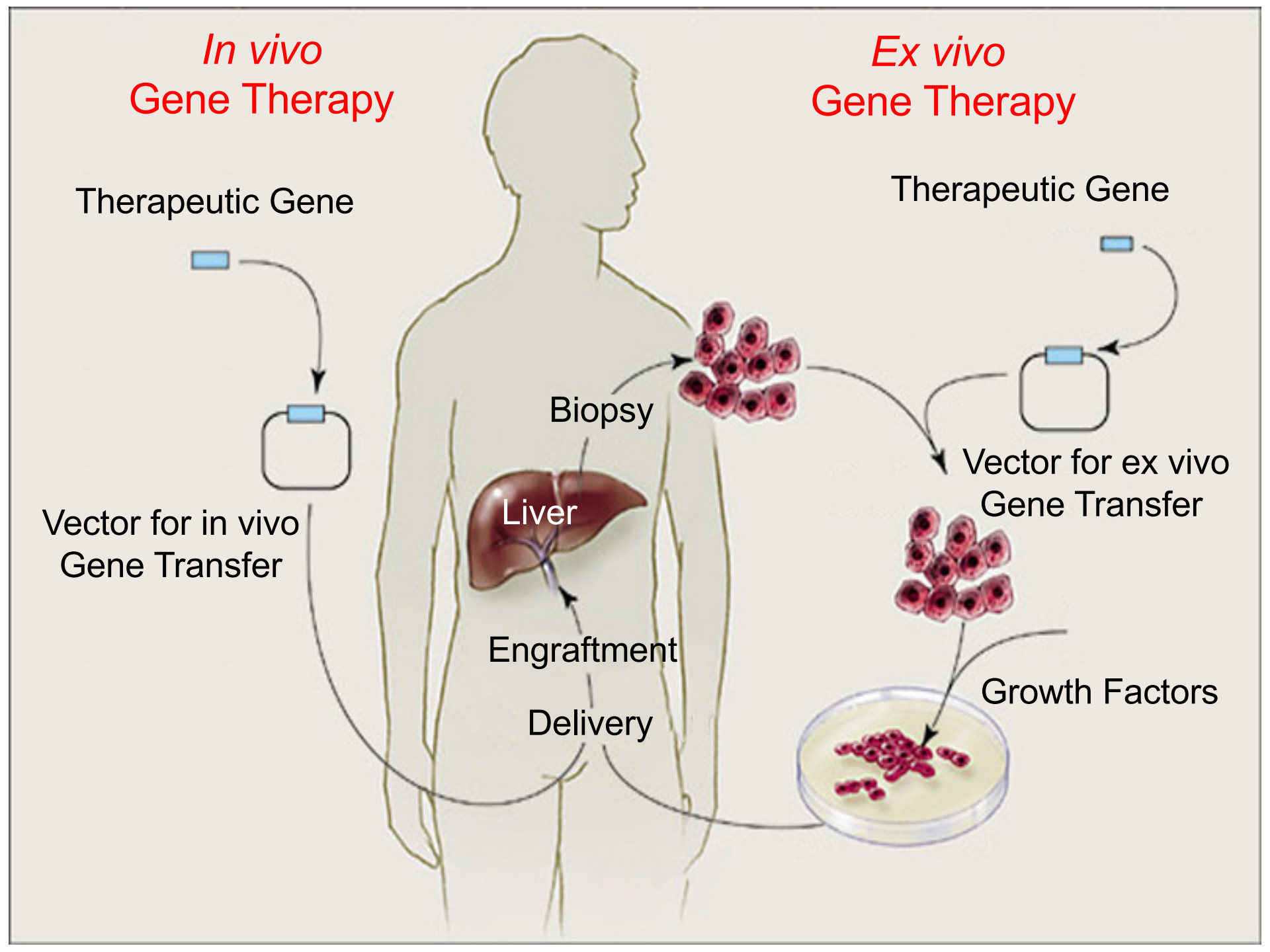
3. Tumori

4. Malattie virali: AIDS

Terapia Genica

***In vivo*: introduzione di informazione genetica, sotto forma di un vettore virale o altro, direttamente nel paziente**

***Ex vivo*: introduzione di informazione genetica, sotto forma di un vettore virale o altro, in cellule o tessuti prelevati da un paziente e successivamente re-impiantati**


In vivo
Gene Therapy
Ex vivo
Gene Therapy
Therapeutic Gene
Therapeutic Gene
Biopsy
Liver
Vector for in vivo
Gene Transfer
Vector for ex vivo
Gene Transfer
Engraftment
Delivery
Growth Factors

Il successo della terapia genica è basato su:

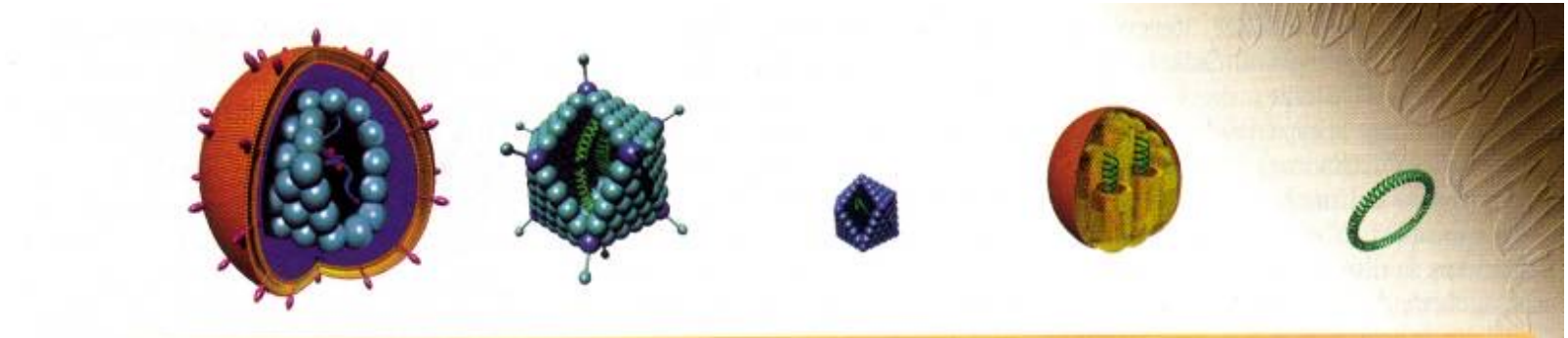
- Efficiente trasferimento genico nella cellula bersaglio
- Appropriato livello di espressione del transgene
- Persistenza dell'espressione genica

  Regolazione dell'espressione genica
- Tolleranza immunitaria verso il prodotto del transgene
- Bio-sicurezza

TRASFERIMENTO GENICO

- VETTORI NON VIRALI: DNA "nudo", DNA coniugato con poli-lisina, lipidi cationici
- VETTORI VIRALI: retrovirus (MoMLV), adenovirus, virus adeno-associati, virus erpetici, lentivirus

VETTORI PER LA TERAPIA GENICA



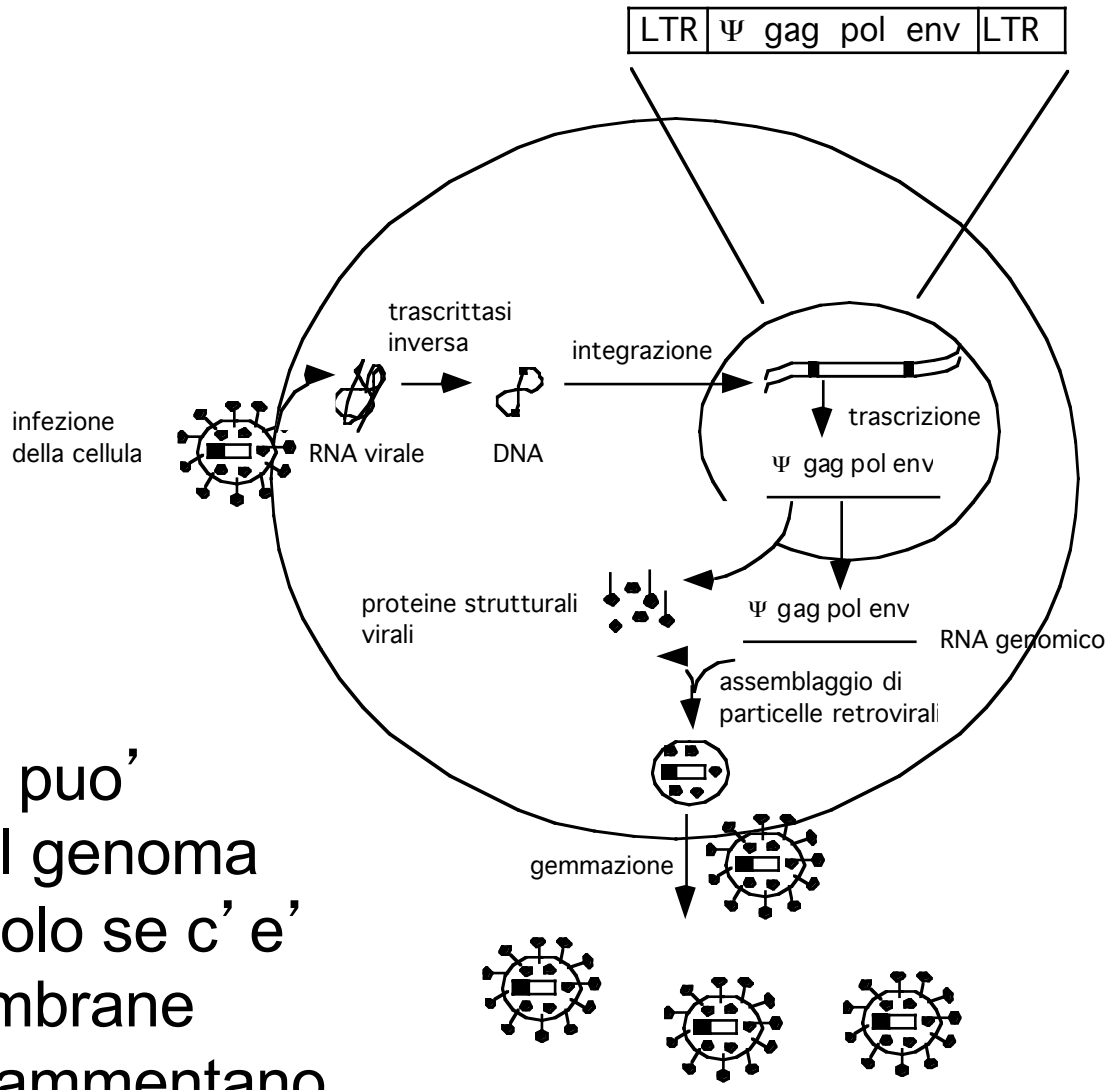
	Retrovirus	Adenovirus	Virus adeno--associati	Liposomi	DNA «nudo»
Alcuni potenziali vantaggi	Integrano i geni nei cromosomi dell'ospite, consentendo una stabilità a lungo termine	La maggior parte non causa gravi malattie; possono accogliere geni estranei di grandi dimensioni	Integrano i geni nei cromosomi dell'ospite; non causano malattie umane note	Non hanno geni virali e pertanto non causano malattie	Come i liposomi; si prevede che sia utile per le vaccinazioni
Alcuni difetti dei vettori esistenti	I geni si integrano a caso, pregiudicando a volte i geni dell'ospite; molti infettano solo cellule in divisione	I geni a volte funzionano transitoriamente, per la mancata integrazione o l'attacco del sistema immunitario	Non possono accogliere geni estranei di grandi dimensioni	Sono meno efficienti dei virus nel trasferire geni alle cellule	È inefficiente nel trasferimento genico e instabile in gran parte dei tessuti dell'organismo

Slim Films

VETTORI RETROVIRALI (MoMLV)

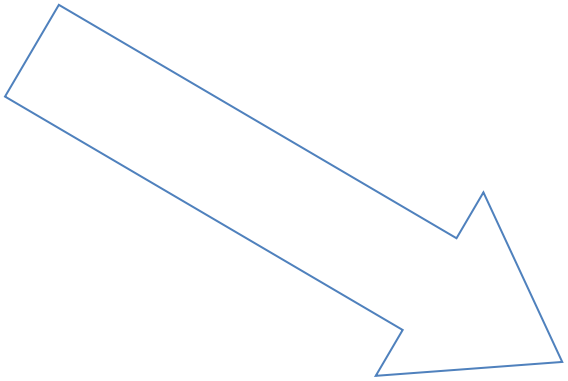
- VANTAGGI: elevata efficienza di infezione; integrazione nel genoma della cellula target ed espressione permanente del transgene; stabilità strutturale
- DIMENSIONE DEL TRANSGENE: <7 kb
- SVANTAGGI: mutagenesi inserzionale, incapacità di infettare cellule quiescenti

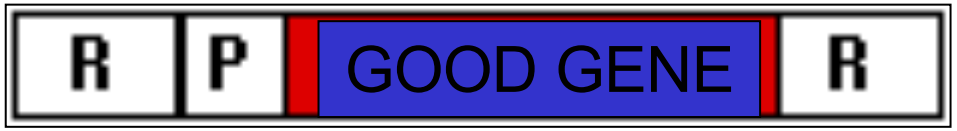
Ciclo replicativo di un retrovirus



Il DNA virale puo' integrarsi nel genoma dell' ospite solo se c' e' mitosi-> membrane nucleari si frammentano

"Tamed" virus N
nothing more than "good gene",
packaging signal and repeats



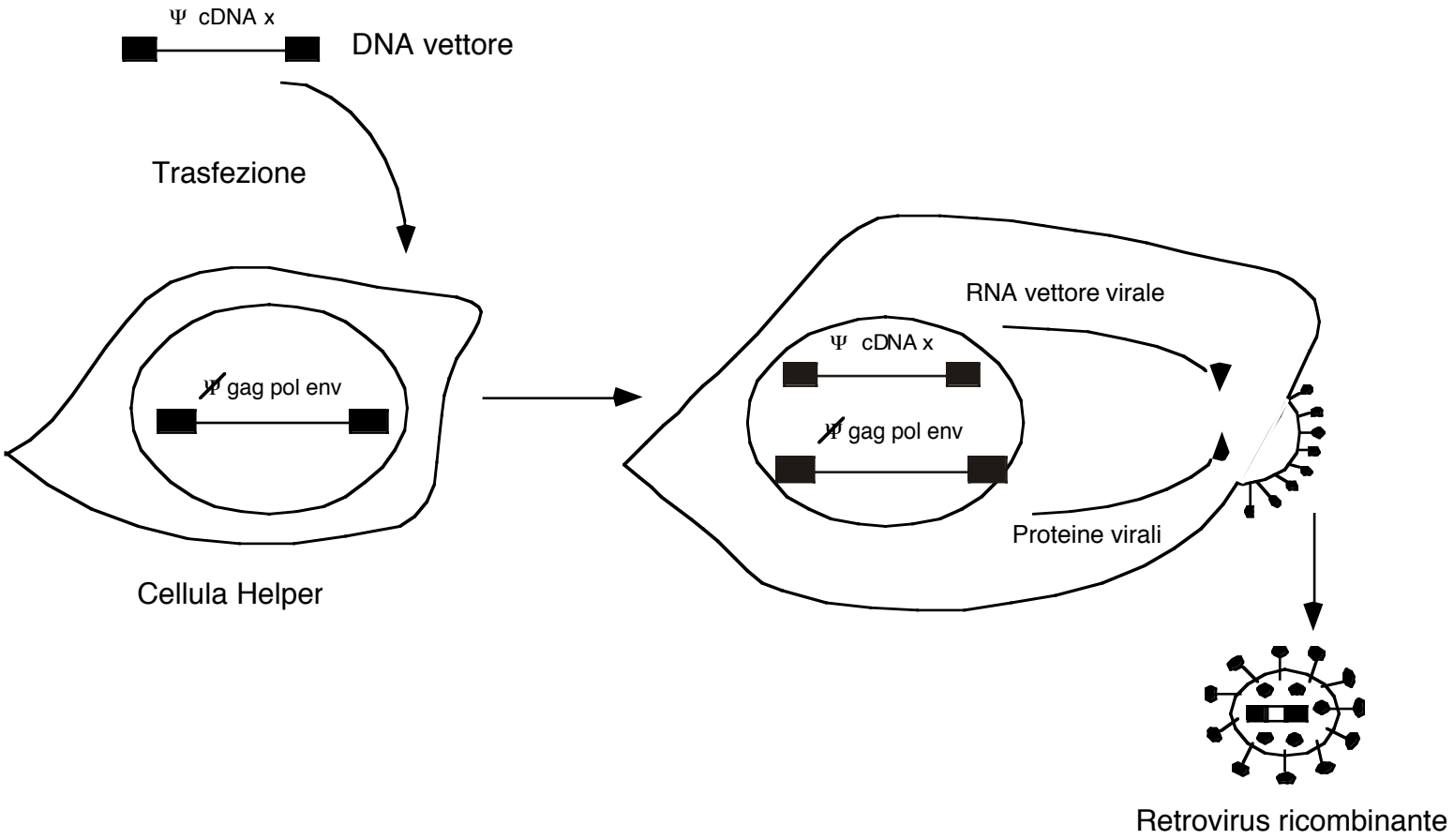


This virus go in our cells

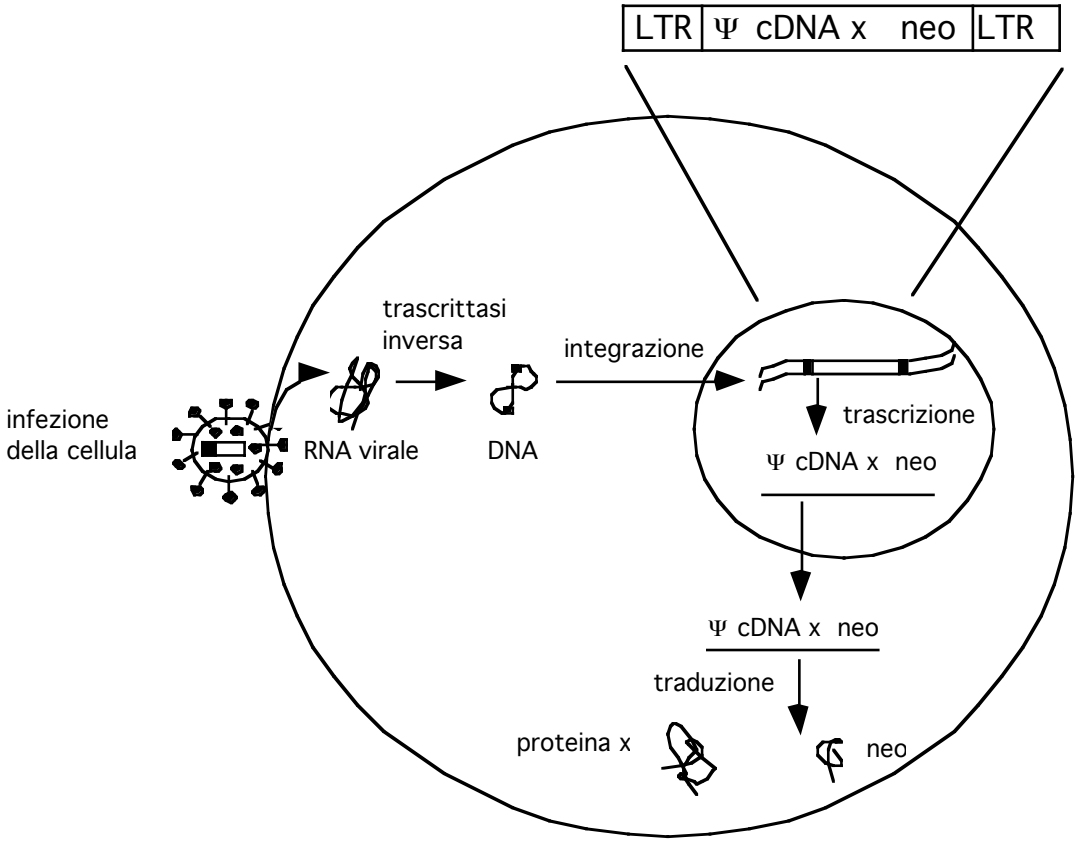
Helper virus – not able to pack himself;
allow replication of "tamed" virus

Helper virus
helps to create
suitable amount
of "tamed" virus
in cell culture

Produzione di retrovirus ricombinante



Infezione della cellula bersaglio



VETTORI LENTIVIRALI (HIV)

- VANTAGGI: presenta gli stessi vantaggi dei retrovirus ed è in grado di infettare anche cellule quiescenti
- DIMENSIONE DEL TRANSGENE: 7-10 Kb
- SVANTAGGI: possibilità di indurre mutagenesi inserzionale

Lentiviral vectors

Lentiviruses are retroviruses
that can infect both dividing and nondividing cells

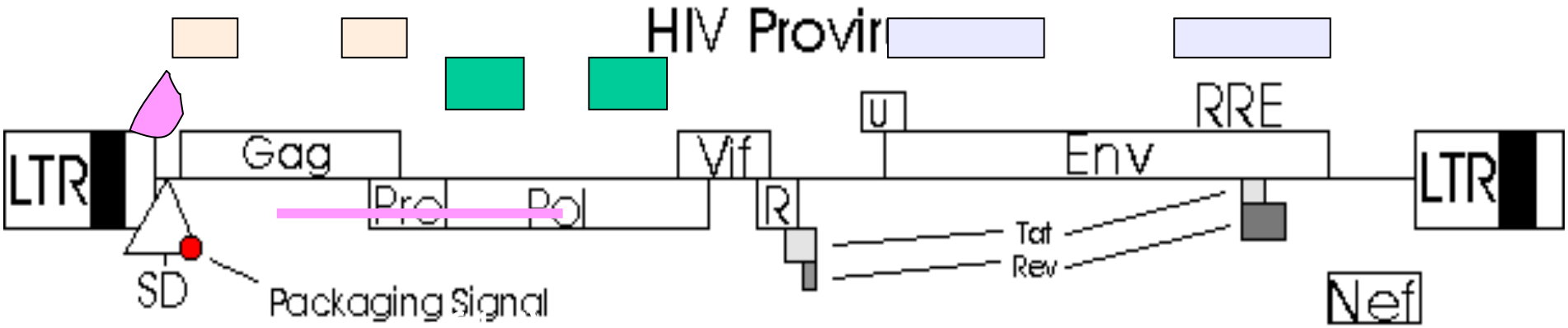
Preintegration complex of lentiviruses can get through the intact membrane of the nucleus of the target cell.

Able to infect nondividing or terminally differentiated cells such as neurons, macrophages, hematopoietic stem cells, retinal photoreceptors, and muscle and liver cells

Example of lentiviruses:
HIV-1 (infects T-helper cells) – AIDS.

Good feature – no immune response!

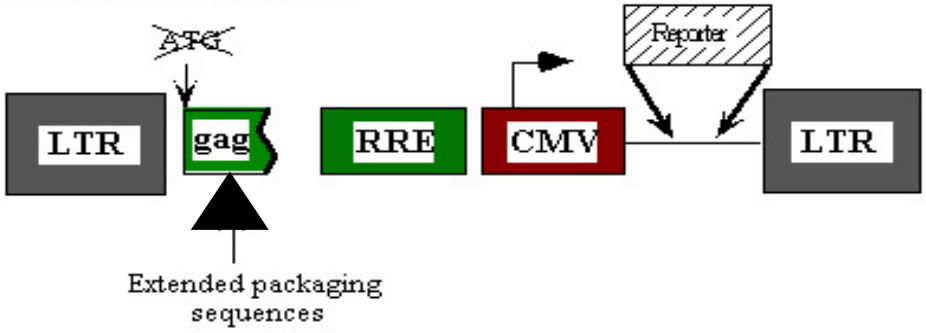
Genes encoded by HIV-1 virus



1. Latent stage (virus integrated but not replicate itself)
2. Virus gradually infects helper T cells
3. Production of infective viral particles that are released into the blood by the host cell lysis to infect other cells

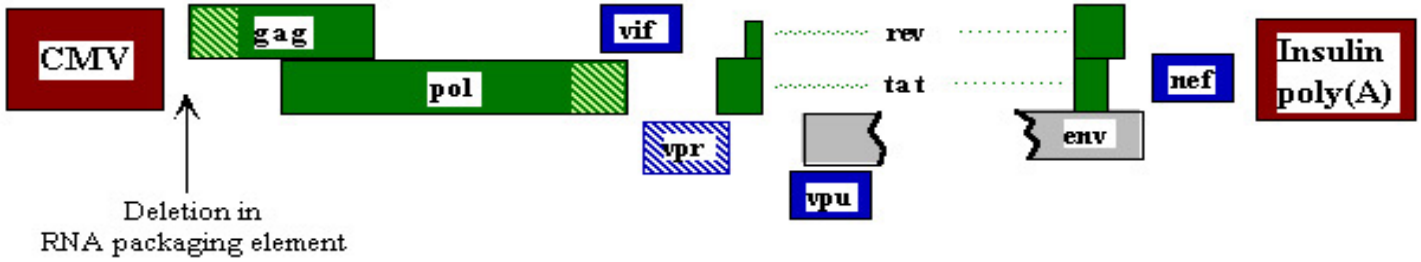
HIV Vectors made in a three Plasmid Expression System

Transfer construct



the original envelop proteins and *gag* sequence promoter removed, gag needed just as pack signal.

Packaging construct

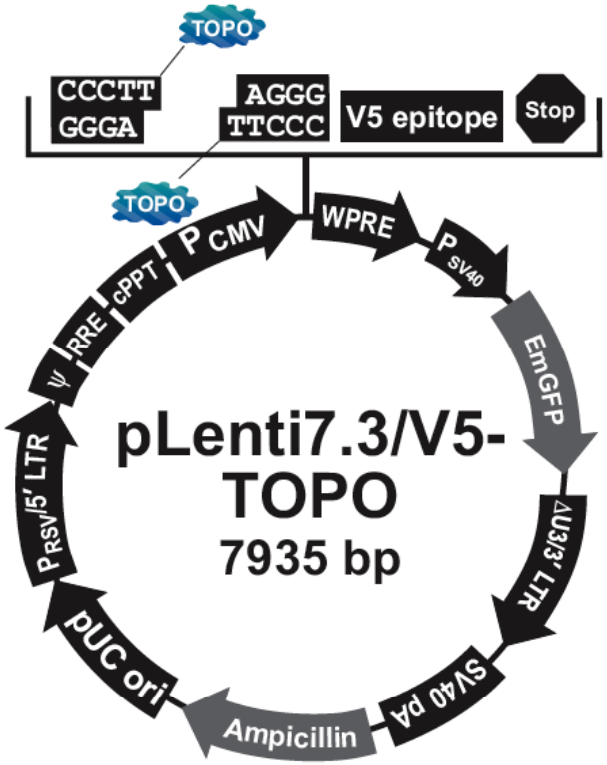


All except envelope and LTRs

Envelope construct

To move it from T-cell specific to broad range or change specificity

All three vectors are introduced to packaging cell line



Comments for pLenti7.3/V5-TOPO
7935 nucleotides

RSV/5′ LTR hybrid promoter: bases 1-410
    RSV promoter: bases 1-229
    HIV-1 5′ LTR: bases 230-410
HIV-1 psi (ψ) packaging signal: bases 521-565
HIV-1 Rev response element (RRE): bases 1075-1308
cPPT: bases 1801-1923
CMV promoter: bases 1935-2519
TOPO® cloning site: bases 2558-2567
V5 epitope: bases 2630-2671
WPRE: bases 2690-3287
SV40 promoter: bases 3298-3606
EmGFP: bases 3665-4384
ΔU3/3′ LTR: bases 4455-4689
    ΔU3: bases 4455-4508
    3′ LTR: bases 4509-4689
SV40 polyadenylation signal: bases 4761-4892
bla promoter: bases 5751-5849
Ampicillin (*bla*) resistance gene: bases 5850-6710
pUC origin: bases 6855-7528

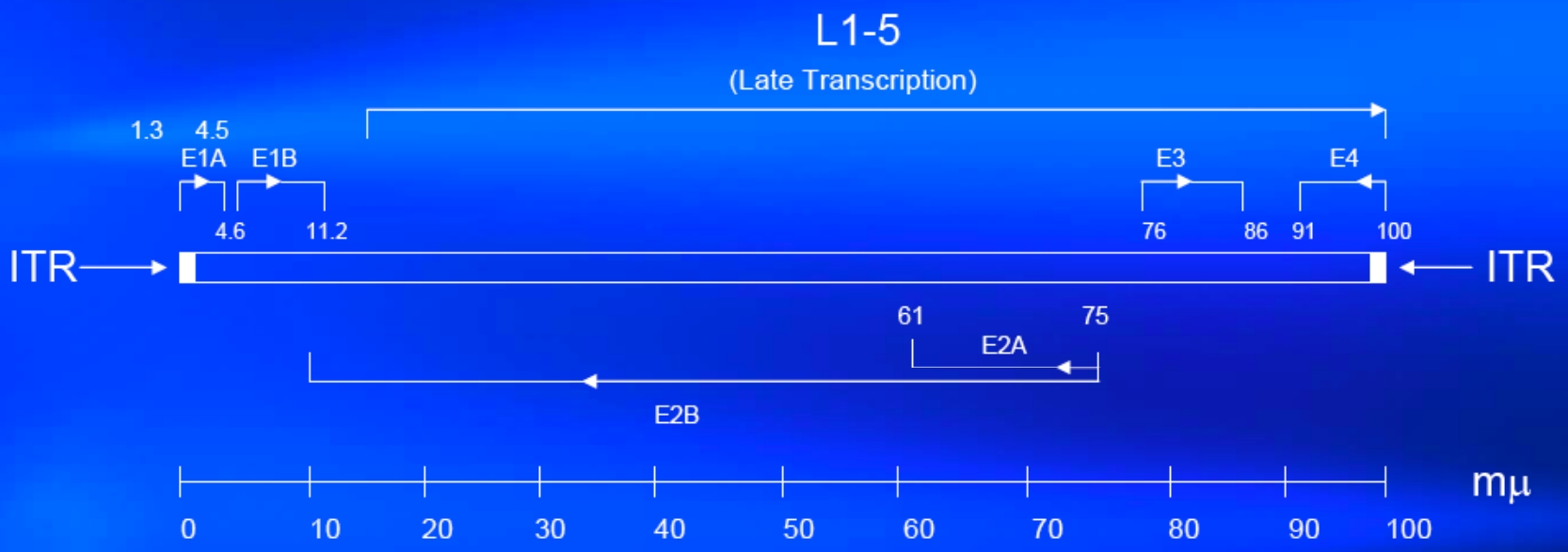
VETTORI ADENOVIRALI (AdV)

- VANTAGGI: elevata efficienza di infezione; infettano anche cellule quiescenti (fegato e muscolo); applicazione per terapia genica *in vivo*

- DIMENSIONE DEL TRANSGENE: 8-35 Kb

- SVANTAGGI: tossicità acuta: risposta immune, dose-dipendente, contro le proteine del capside; non integrano il transgene nel genoma della cellula target; difficoltà nella produzione su larga scala

Adenoviral 5 (Ad5) Genome



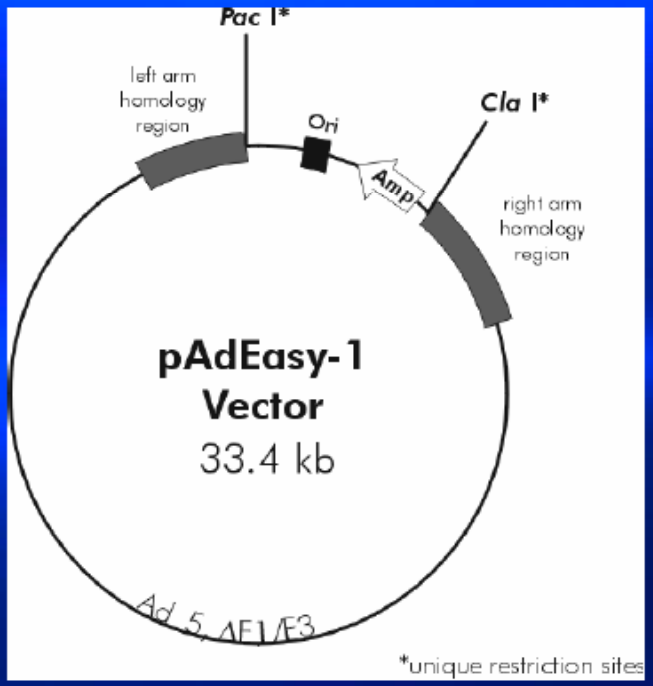
100mμ=36kb

E1: Early activation proteins
E2: Adenoviral DNA replication
E3: Prevent Antiviral immune response
E4: Transcriptional control, DNA replication, shut-off of host macromolecular synthesis, viral chromosome synthesis

Late Genes: Viral structural proteins: capsid proteins, hexon, penton and fiber. All are transcribed from major late promoter.

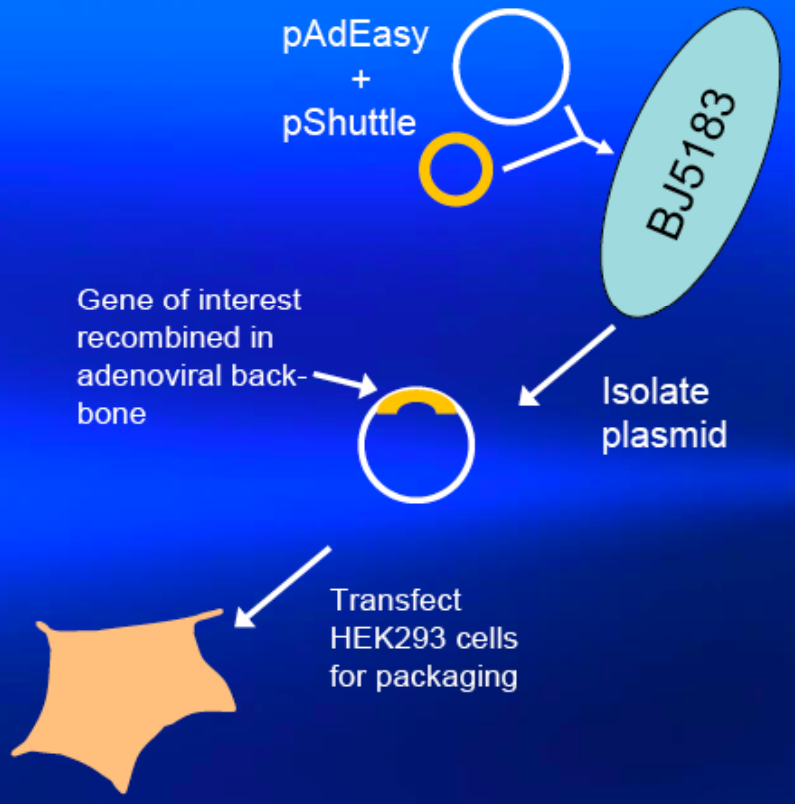
Replication defective adenovirus

- Remove sequence in vector DNA for viral replication
  - E1, E3 genes
- Package virus in E1 complementing cell line (HEK293)
- Vector DNA does not contain necessary viral proteins for replication
  - Limited pathogenicity

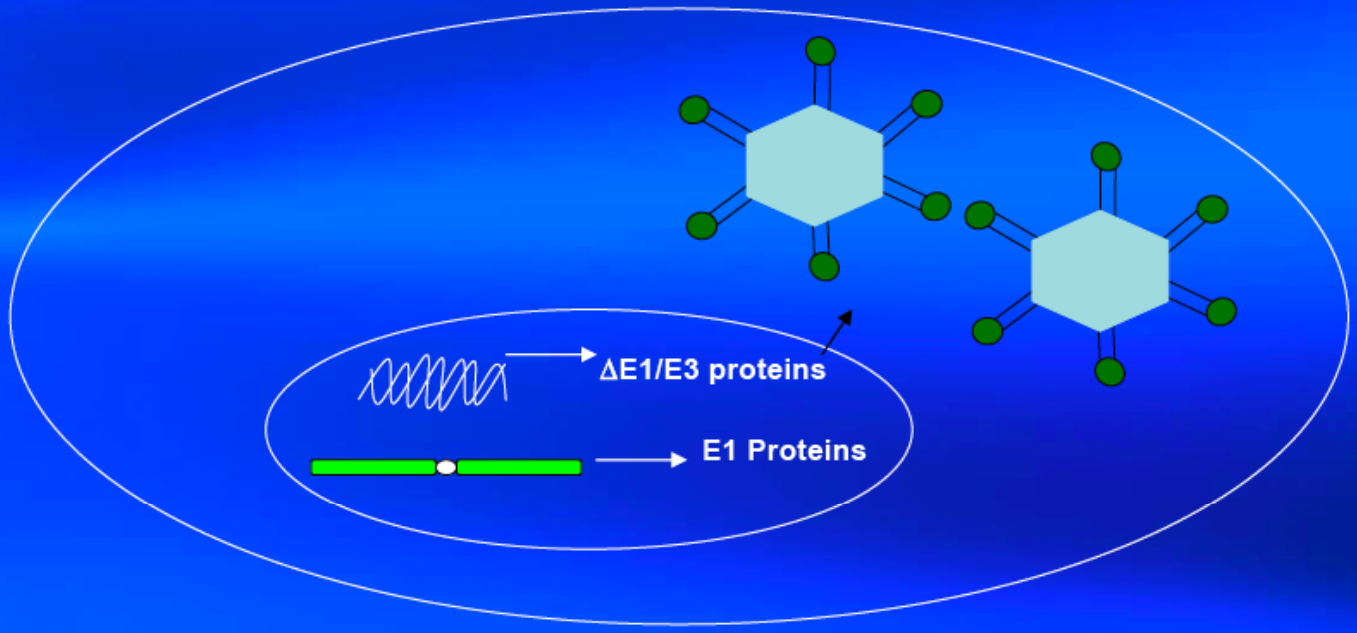


Recombination in *E. coli* The AdEasy™ System

- Clone gene of interest into shuttle vector
- Transform shuttle and pAdEasy-1 (adenoviral backbone) into BJ5183 *E. coli*
- Isolate recombinants
- Package virus in HEK293 cells

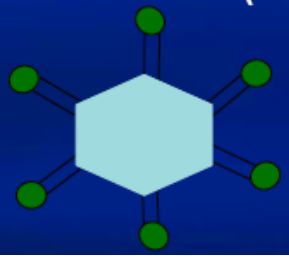


Viral Packaging with the AdEasy™ Adenoviral Vector System



Ad 293 Cell

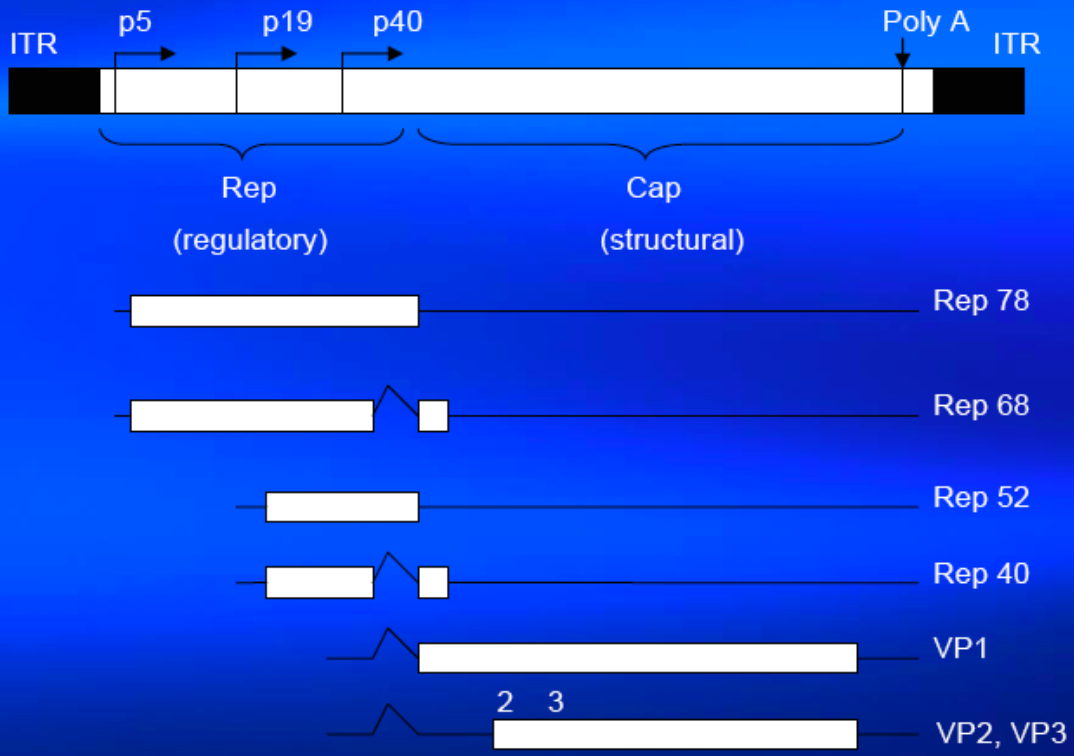
Lyse open cell to obtain virus
(Adenovirus is NOT a budding virus)



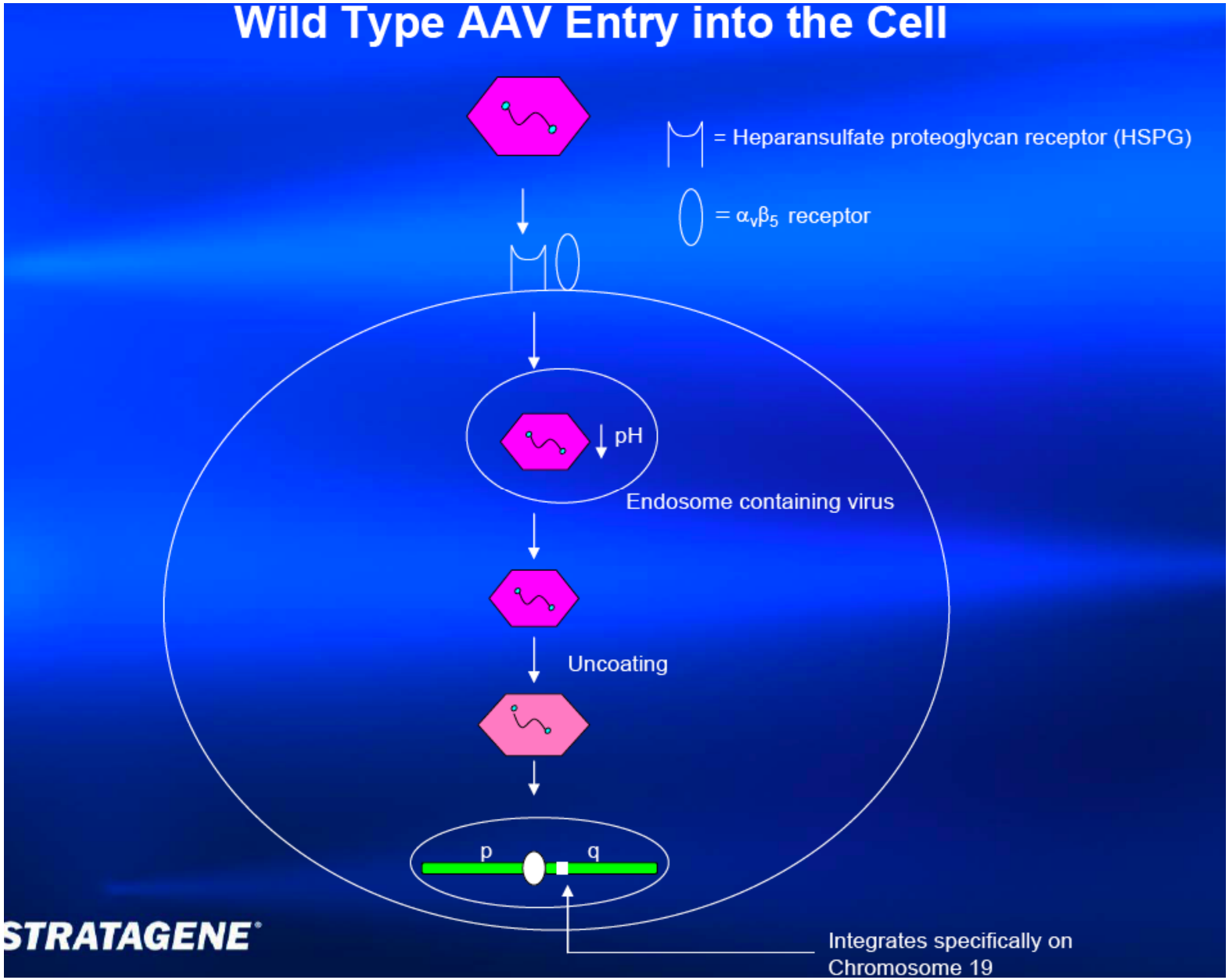
VETTORI ADENO-ASSOCIATI (AAV)

- VANTAGGI: infettano anche cellule quiescenti, danno integrazione sito-specifica nel cromosoma 19 umano, mediata dalla proteina rep; applicazione per terapia genica *in vivo*
- SVANTAGGI: sono in grado di veicolare nel proprio genoma solo geni molto piccoli (max 4-5 Kb); bassa efficienza di produzione di particelle infettanti

AAV Genome



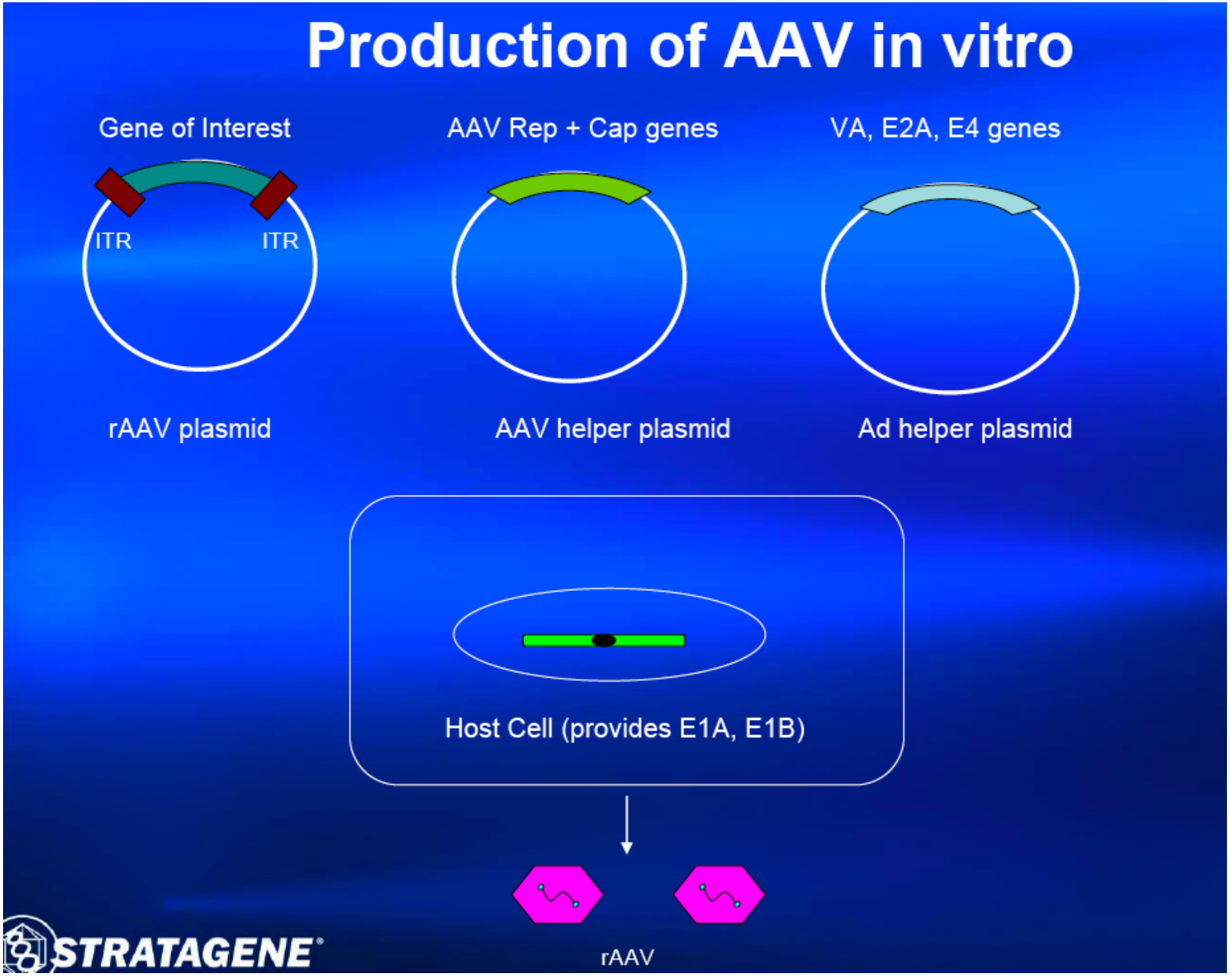
*AAV is a replication defective virus: Dependent on Adenovirus for replication


Wild Type AAV Entry into the Cell
= Heparansulfate proteoglycan receptor (HSPG)
= $\alpha_v\beta_5$ receptor
pH
Endosome containing virus
Uncoating
p
q
STRATAGENE
Integrates specifically on
Chromosome 19

Recombinant AAV Applications

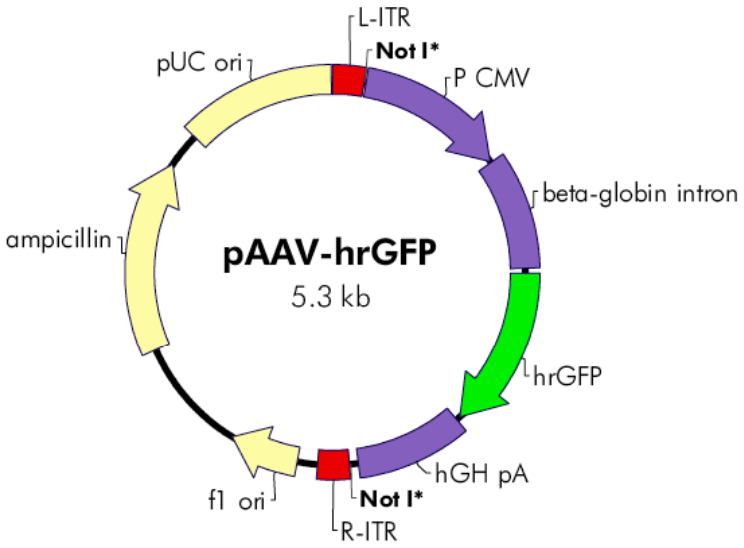
- Gene delivery to difficult-to-transfect cells
- Express mammalian proteins
  - Long-term protein expression in slowly dividing or non-dividing cells
  - Can use clonal expansion to isolate stably-expressing cell population
- Gene Therapy
  - High titers and ability to infect non-dividing cells

Production of AAV in vitro



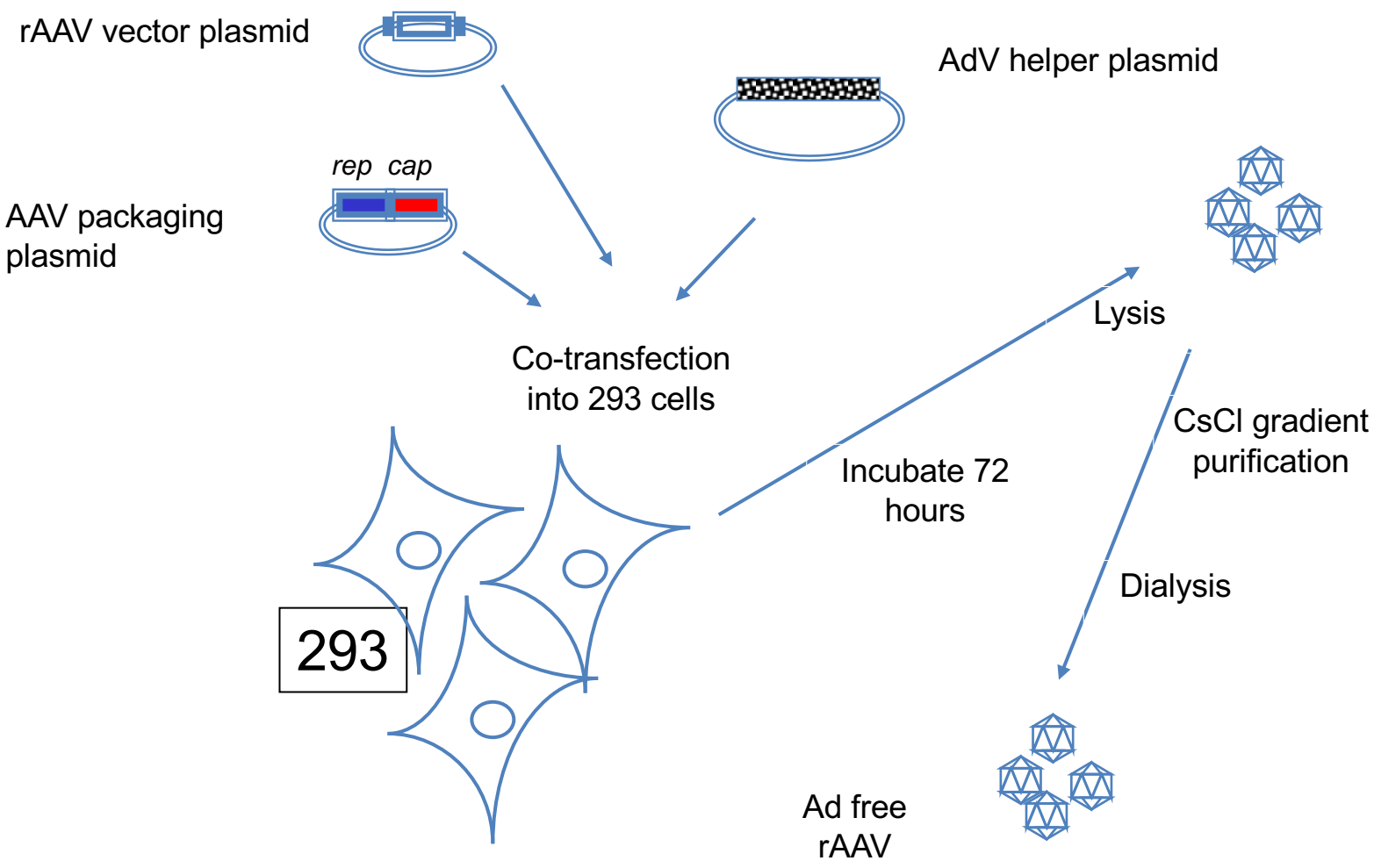
Organization of AAV vectors

left inverted terminal repeat 1–141
CMV promoter 150–812
β-globin intron 820–1312
hrGFP ORF 1335–2051
hGH polyA 2070–2548
right inverted terminal repeat 2588–2728
f1 origin 2820–3126
ampicillin resistance (*bla*) ORF 3645–4499
pUC origin 4653–5320



*Non-unique sites used to release the expression cassette

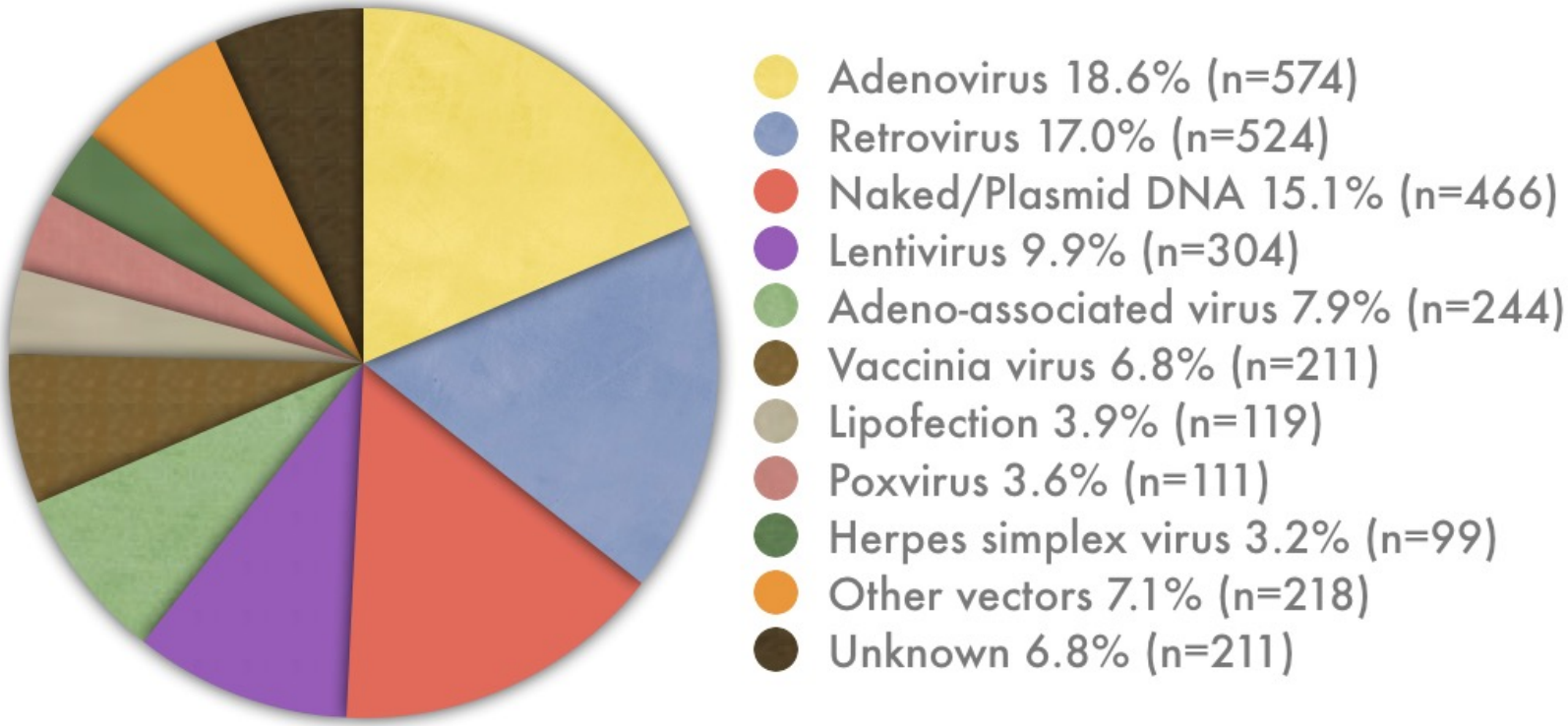
Ad free rAAV Production



VETTORI ERPETICI (HSV)

- VANTAGGI: infettano anche cellule quiescenti
- DIMENSIONE DEL TRANSGENE: 40 Kb
- SVANTAGGI: esprimono proteine virali tossiche; sono immunogenici; non integrano il transgene nel genoma della cellula target; espressione genica di breve durata

Vectors Used in Gene Therapy Clinical Trials



Gli obiettivi della terapia genica

1. Malattie genetiche: immunodeficienze, emofilia, talassemia, distrofia muscolare, genodermatosi, malattie lisosomiali, fibrosi cistica

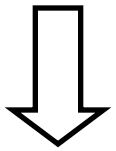
2. Malattie metaboliche e autoimmuni: diabete, sclerosi multipla

3. Tumori

4. Malattie virali: AIDS

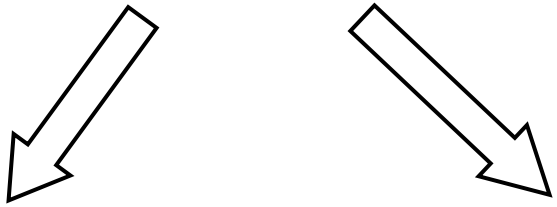
Malattia	Gene trasferito	Tessuto bersaglio
ß-talassemia	ß-globina	Midollo osseo
Immunodeficienza congenita (SCID)	ADA	Midollo osseo/linfociti
Distrofia muscolare	Distrofina	muscolo
Fibrosi cistica	CFTR	Epitelio alveolare
Emofilia A e B	Fattore VIII e IX	Epatociti/muscolo
Morbo di Gaucher	Glucocerebrosidasi	Midollo osseo
Mucopolisaccaridosi tipo I	a-L-iduronidasi	fibroblasti
Mucopolisaccaridosi tipi VII	ß-glucuronidasi	Midollo osseo/CNS
Ipercolestelomia familiare	Recettore LDL	fegato
Epidermiolisi bollosa	laminina	cheratinociti

Regenerative Medicine is the process of creating living, functional tissues to repair or replace tissue or organ function lost due to age, disease, damage, or congenital defects.



- the shortage of organs available for donation
- organ transplant rejection

Regenerative Medicine



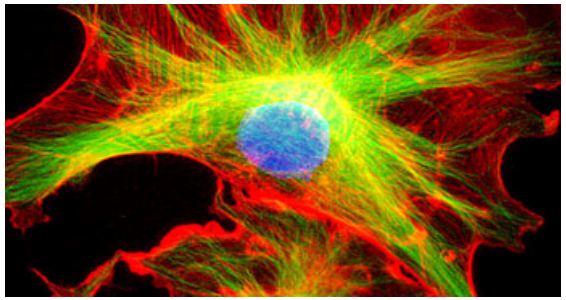
in vitro approach: therapy studied inside the laboratory implanted in the body

in vivo approach: studies performed inside the living body

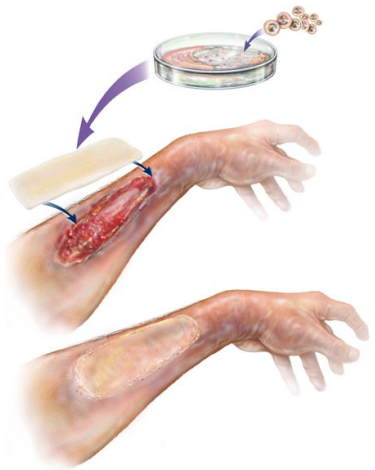
clinical use

Regenerative Medicine

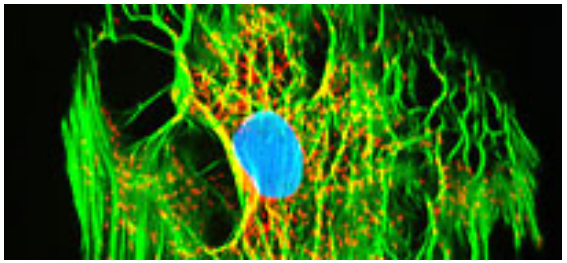
Cell Therapy



Tissue Engineering



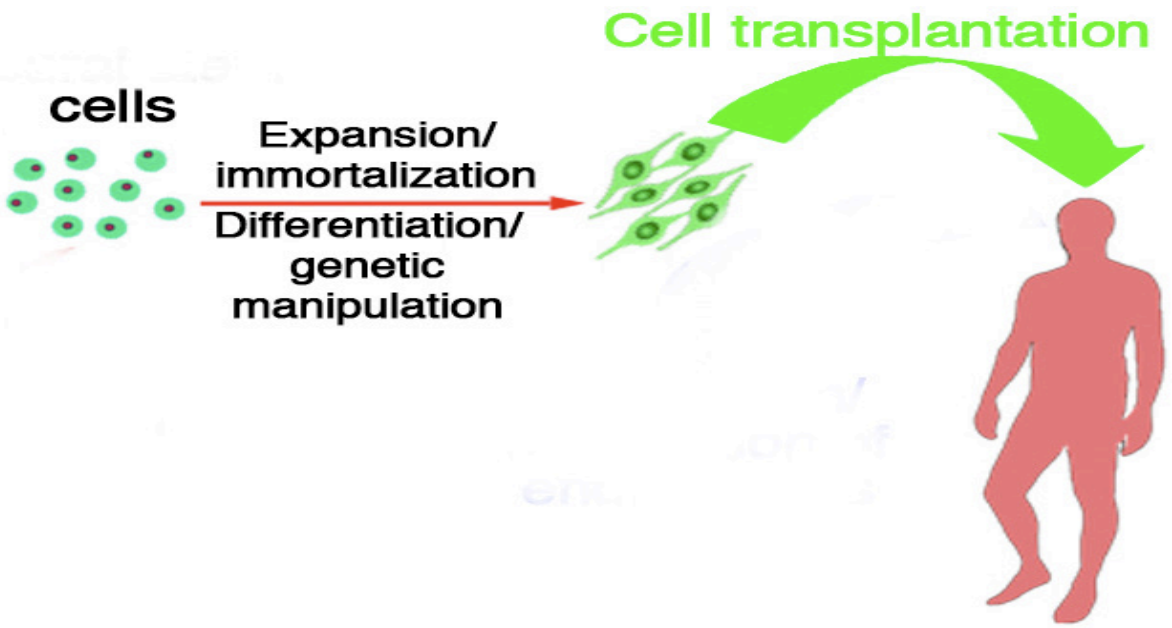
Cell Therapy



There are two ideas behind the use of cells as a medical treatment:

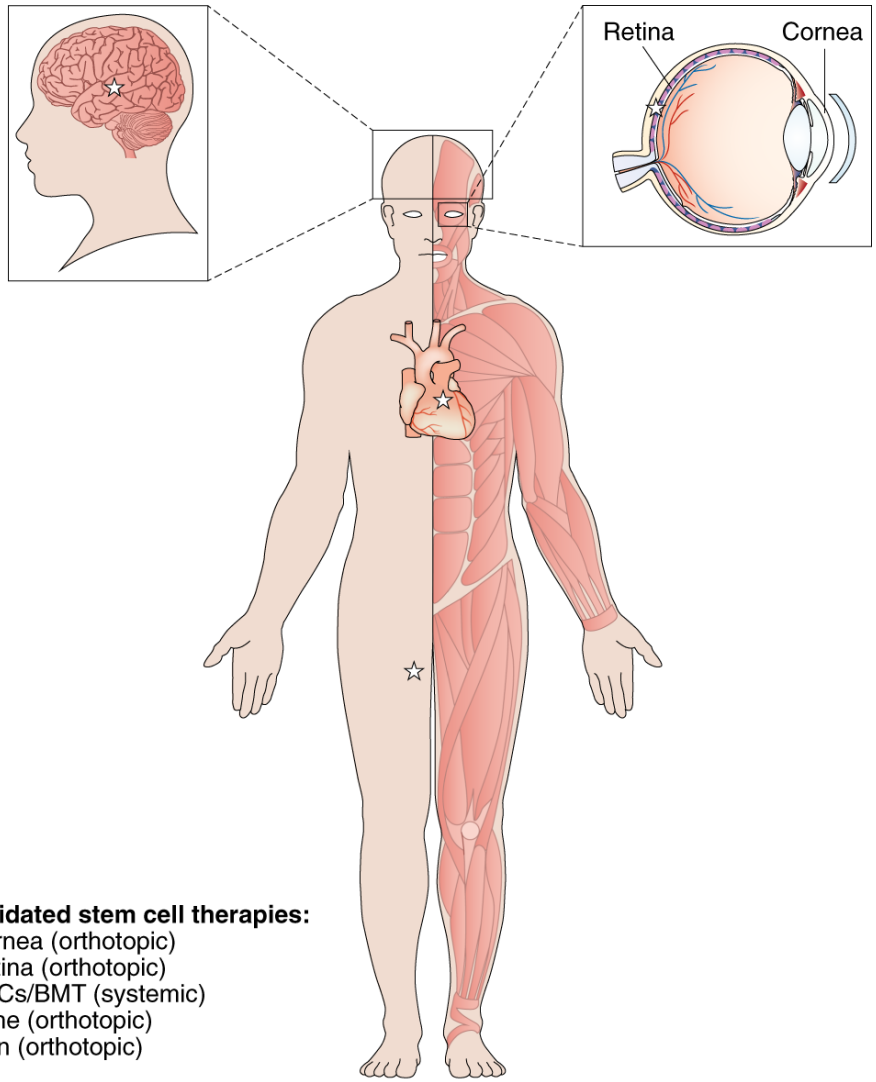
- to provide a source of missing cells
- to manipulate cells to produce a missing substance

Cell Therapy



Transplated cells:

- Mature, functional cells
- Modified human cells
- Transdifferentiated own patient's cells
- Non-human cells (xenotransplantation)
- Stem cells (autologous or allogeneic)



Validated stem cell therapies:
Cornea (orthotopic)
Retina (orthotopic)
HSCs/BMT (systemic)
Bone (orthotopic)
Skin (orthotopic)

Under clinical or preclinical investigation

- Immunomodulation
- Musculoskeletal disorders (muscular dystrophies, bone diseases, joint injuries)
- Cardiovascular diseases (infarct, cardiac failure, peripheral artery diseases)
- Eye diseases
- Neurological disorders (Parkinson's disease, ALS, stroke, multiple sclerosis, spinal cord injury, etc.)
- Diabetes

Terapia Genica

Intervento terapeutico basato sulla modificazione del patrimonio genetico di una cellula somatica, allo scopo di correggere un difetto genetico o di fornire una nuova funzione biologica per combattere una patologia.

ADA-SCID

Malattia autosomica recessiva

Adenosina deaminasi: emzima intracellulare coinvolto nel metabolismo delle purine, che catalizza la conversione dell'adenosina a inosina

Alterazioni nel metabolismo delle purine,
accumulo intracellulare di substrati metabolici, dAXP e adenosina

L'accumulo di substrati metabolici risulta tossico soprattutto per
i linfociti e i loro precursori
Difetto nel differenziamento, nella crescita e nelle funzioni dei linfociti

Assenza di risposta immune umorale e cellulare, infezioni ricorrenti

Il fenotipo dipende dal tipo di mutazione nel gene ADA

Immunodeficienza grave combinata da deficit di adenosina deaminasi (ADA SCID) come modello di terapia genica somatica

La SCID è una malattia letale con solo due alternative terapeutiche disponibili, il trapianto di midollo osseo allogenico e la somministrazione di enzima bovino

Il tessuto bersaglio, cellule emopoietiche, sono facilmente ottenibili e coltivabili in vitro ,prima della re-infusione nel paziente

Il cDNA dell'ADA è molto piccolo (1.5 Kb)

Il gene dell'ADA non richiede una regolazione fine: valori compresi tra il 5% e 50 volte maggiori dell'espressione costitutiva di questo gene non alterano la fisiologia cellulare

Le cellule trasdotte con una copia corretta del gene dell'ADA hanno un notevole vantaggio selettivo di crescita perché riescono ad eliminare più facilmente i metaboliti tossici

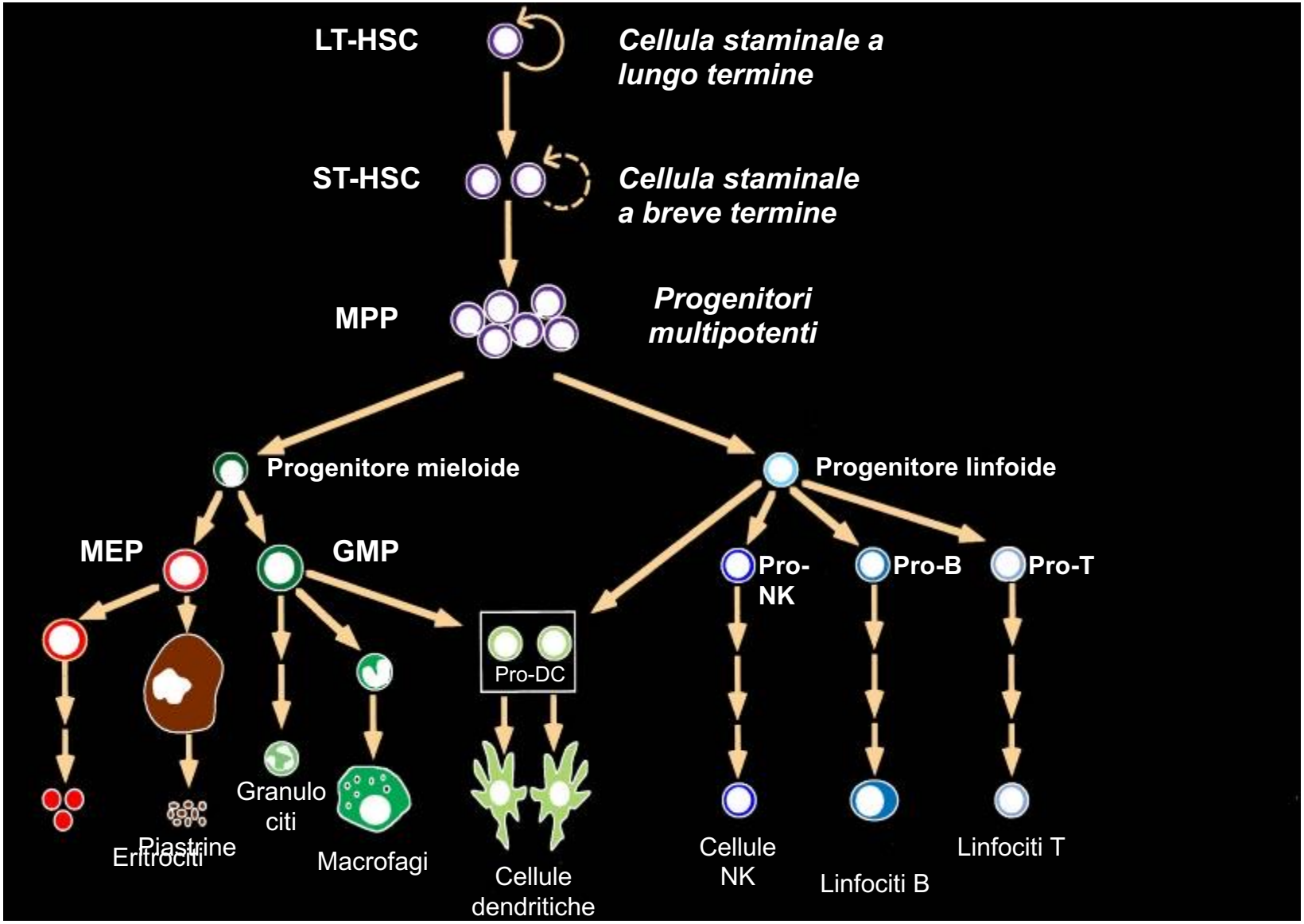
Terapie disponibili per il trattamento dell'ADA SCID

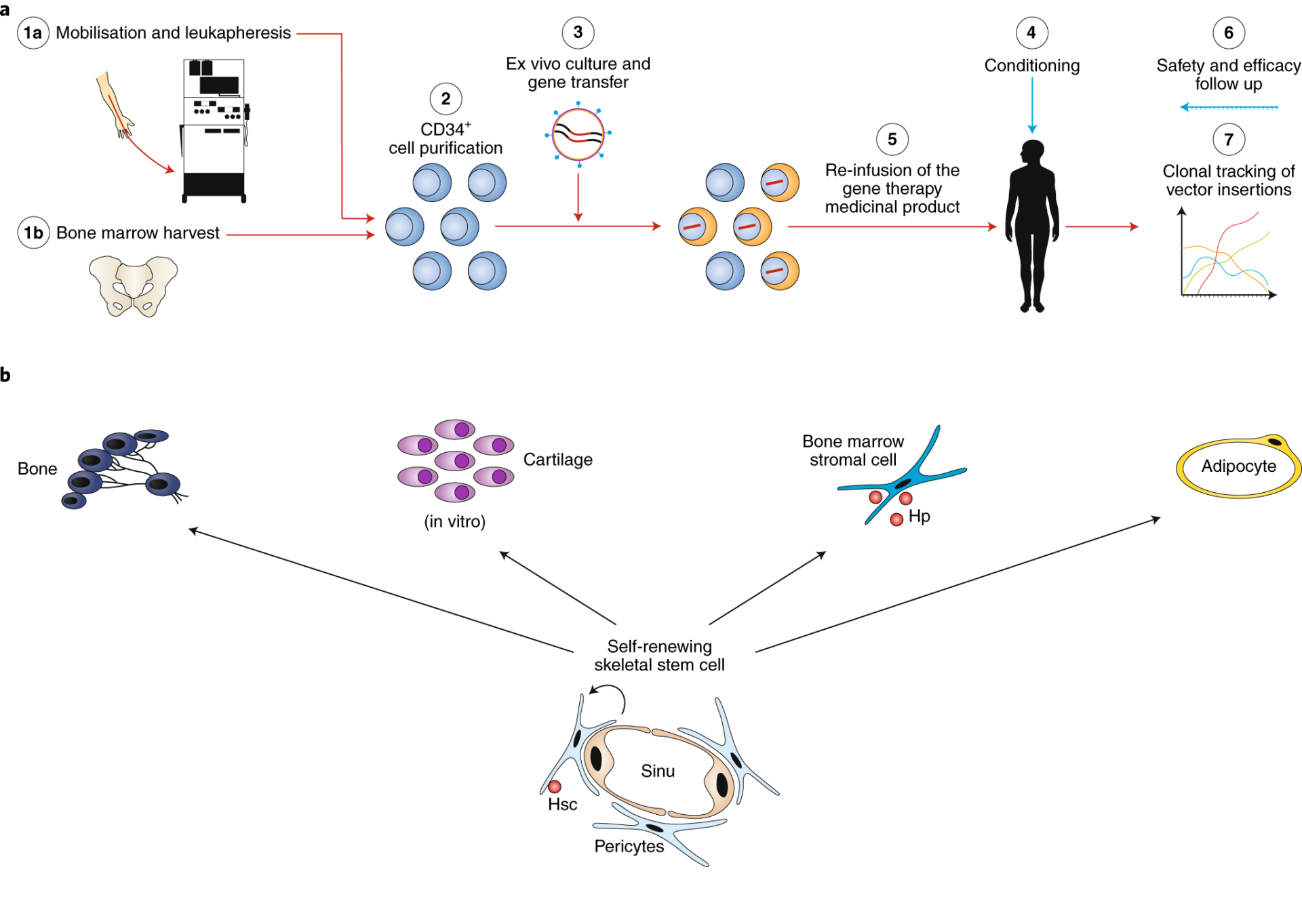
- **Trapianto di midollo**
  - Donatore HLA-identico: cura del 90-100%, disponibile per una minoranza di pazienti
  - Trapianto HLA-non identico: alta morbidità e mortalità

- **Somministrazione di enzima ADA bovino (PEG-ADA)**
  - Corregge le anormalità metaboliche
  - Ripristina in modo variabile le funzioni immuni

- **Terapia genica ex-vivo**
  - Cellule staminali emopoietiche
  - Linfociti

SISTEMA EMOPOIETICO




a
1a Mobilisation and leukapheresis
1b Bone marrow harvest
2
CD34+
cell purification
3
Ex vivo culture and
gene transfer
4
Conditioning
5
Re-infusion of the
gene therapy
medicinal product
6
Safety and efficacy
follow up
7
Clonal tracking of
vector insertions
b
Bone
Cartilage
(in vitro)
Bone marrow
stromal cell
Hp
Adipocyte
Self-renewing
skeletal stem cell
Sinu
Hsc
Pericytes

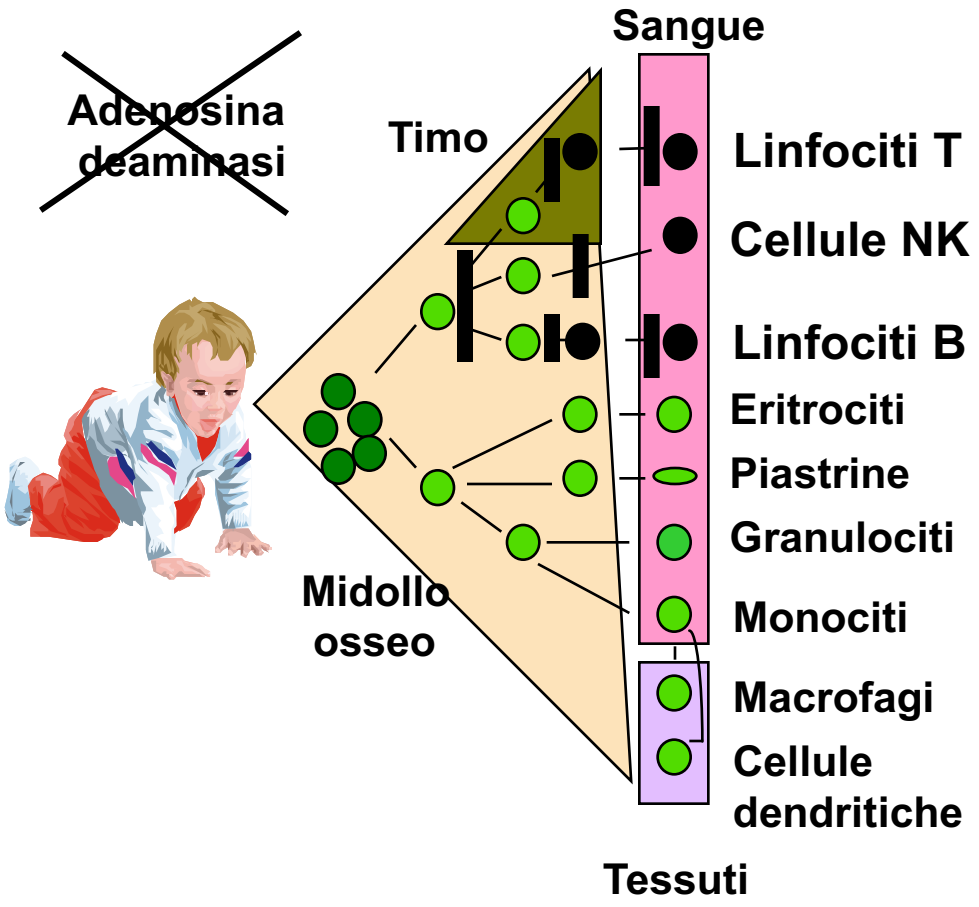
ADA-SCID

•Alterazioni nel metabolismo delle purine, accumulo intracellulare di dAXP e adenosina

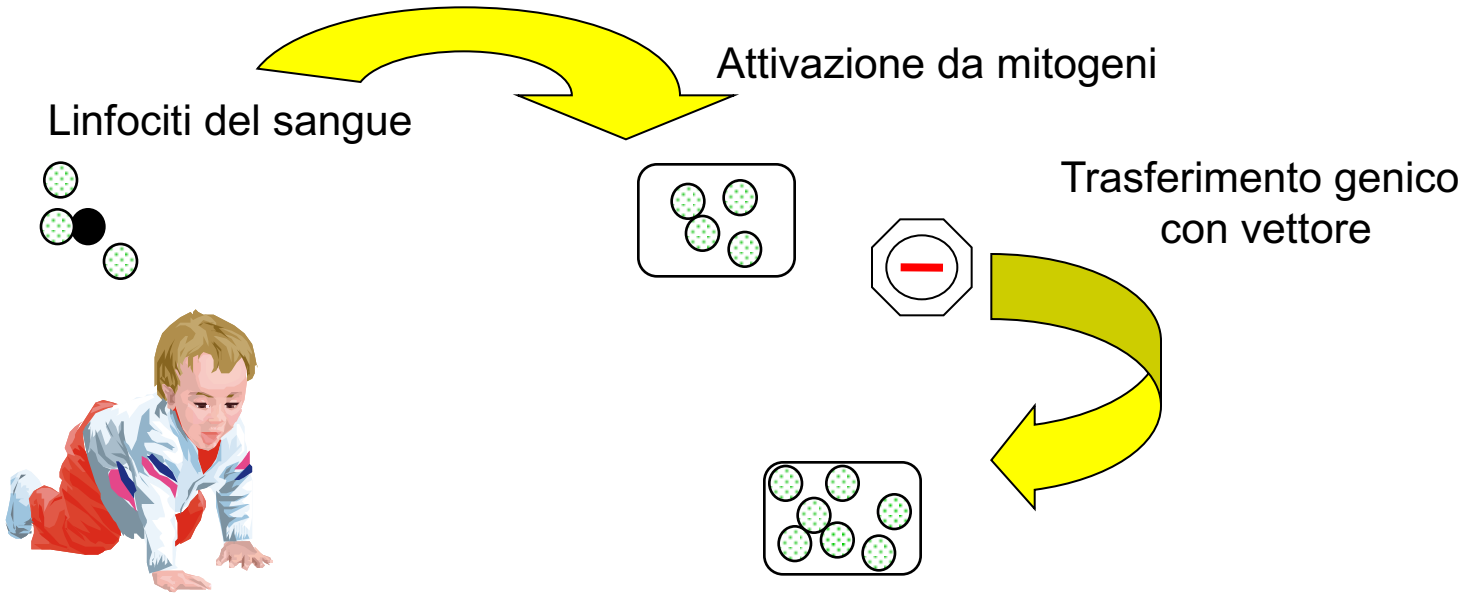
•Difetto nel differenziamento, nella crescita e nelle funzioni dei linfociti

•Assenza di risposta immune umorale e cellulare, infezioni ricorrenti

•Il fenotipo dipende dal tipo di mutazione nel gene ADA

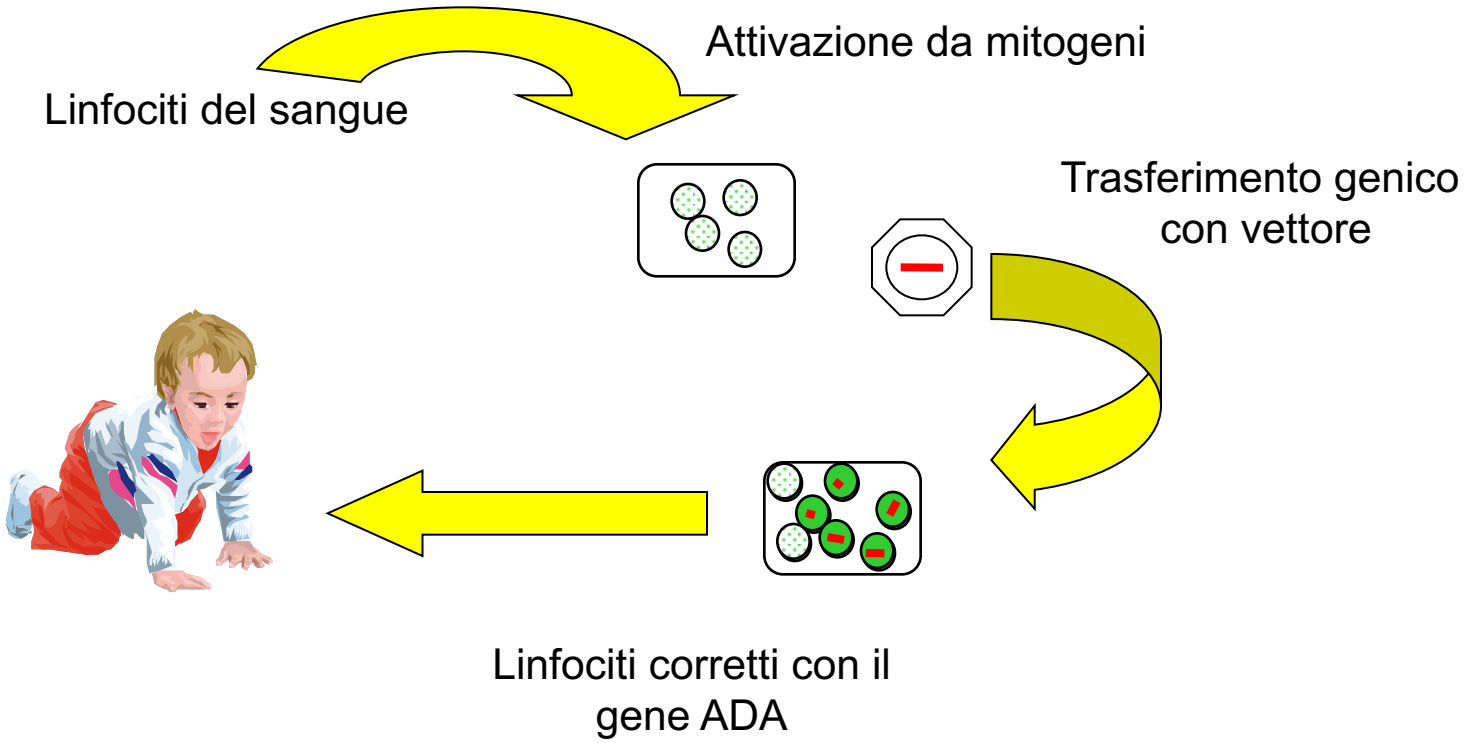


Terapia genica dell'ADA-SCID: protocollo con i linfociti

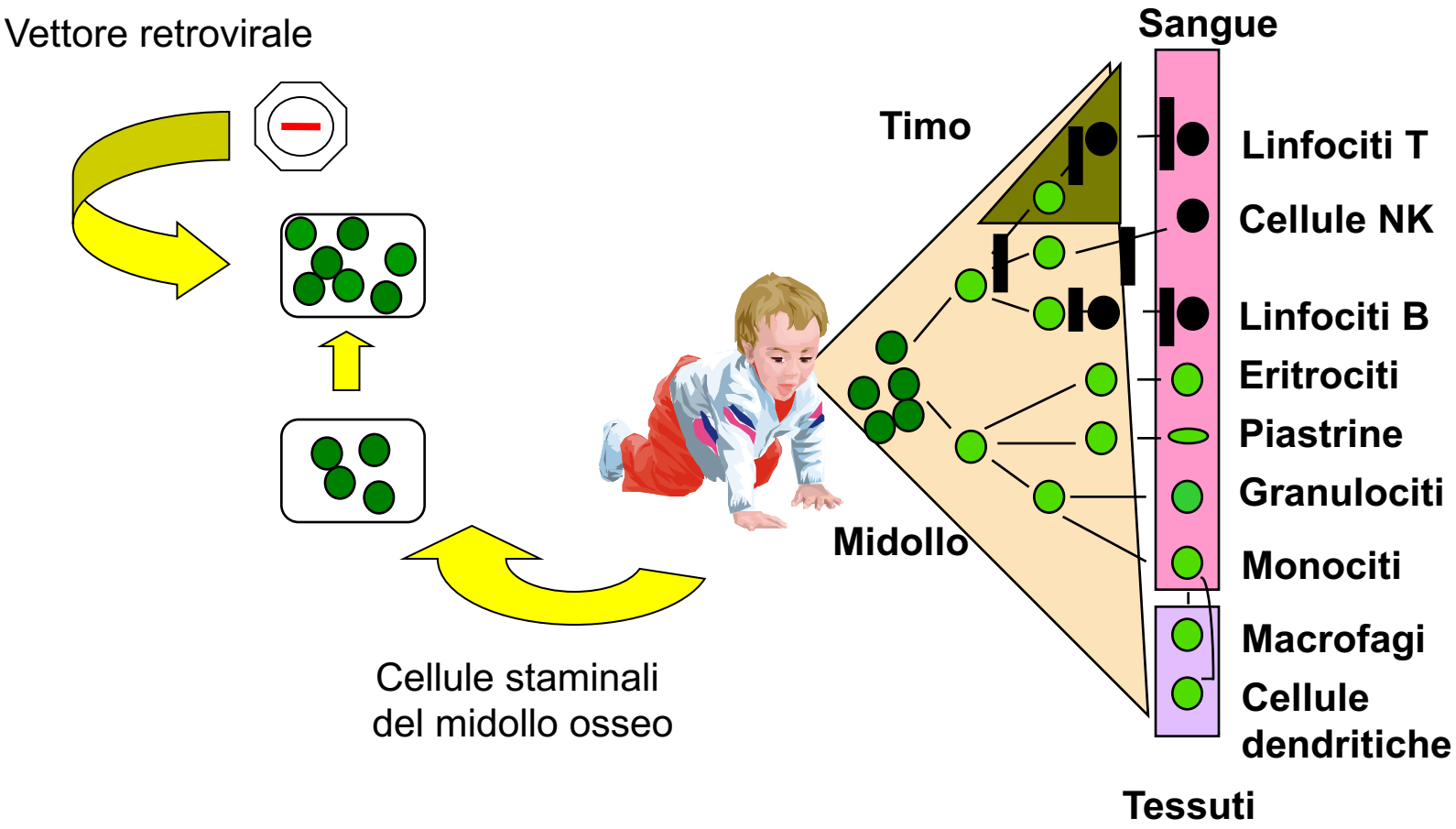


Iniziale risposta alla
terapia sostitutiva

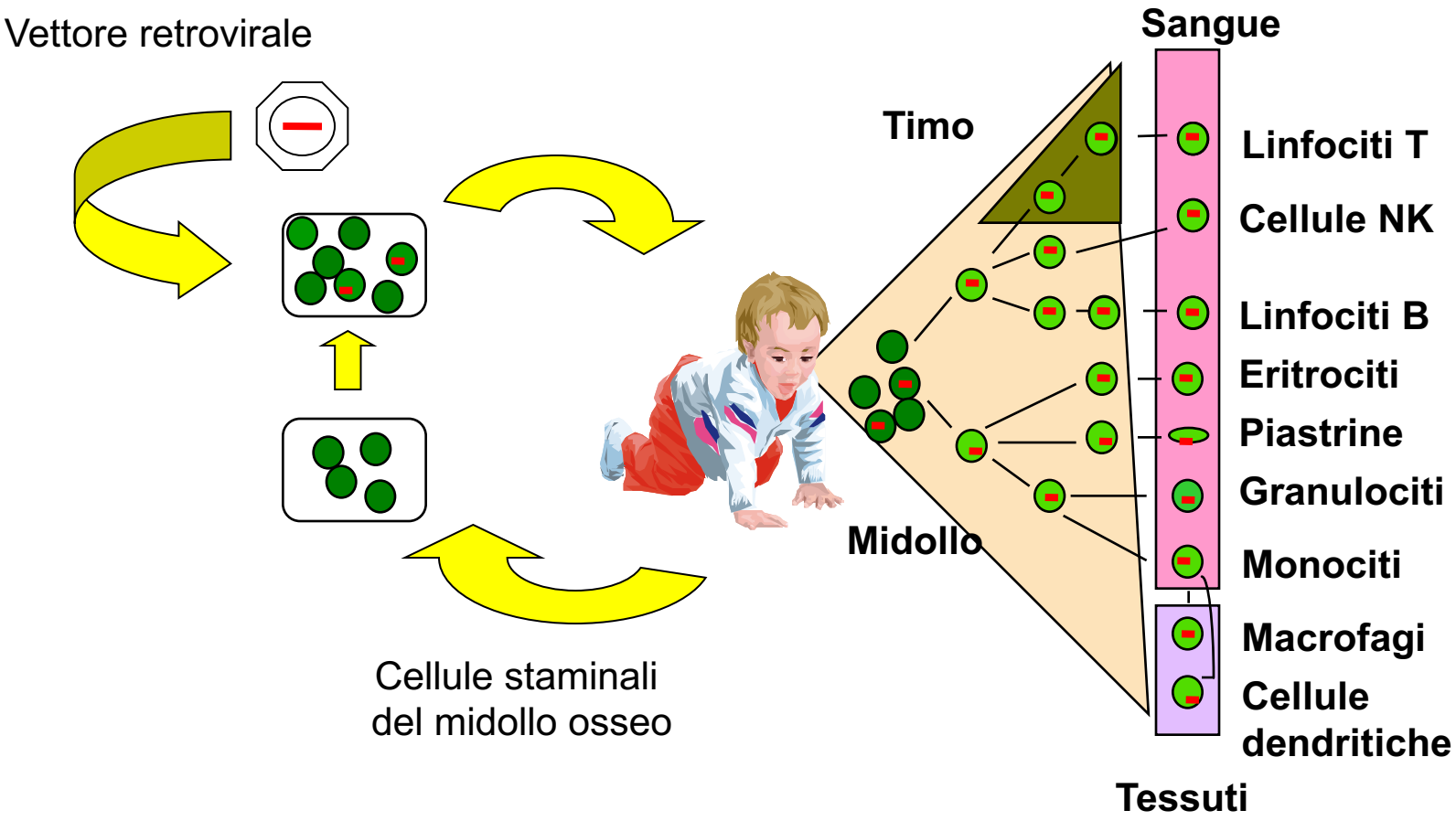
Terapia genica dell'ADA-SCID: protocollo con i linfociti



Terapia genica dell'ADA-SCID: protocollo con cellule staminali



Terapia genica dell'ADA-SCID: protocollo con cellule staminali



Immune reconstitution in ADA-SCID after PBL gene therapy and discontinuation of enzyme replacement.

Aiuti A, Vai S, Mortellaro A, Casorati G, Ficara F, Andolfi G, Ferrari G, Tabucchi A, Carlucci F, Ochs HD, Notarangelo LD, Roncarolo MG, Bordignon C.

Nat Med. 2002 May;8(5):423-5.

Correction of ADA-SCID by stem cell gene therapy combined with non-myeloablative conditioning

Aiuti A, Slavin S, Aker M, Ficara F, Deola S, Mortellaro A, Morecki S, Andolfi G, Tabucchi A, Carlucci F, Marinello E, Cattaneo F, Vai S, Servida P, Miniero R, Roncarolo MG, Bordignon C.

Science 2002 Jun 28;296(5577):2410-3

A serious adverse event after successful gene therapy for X-linked severe combined immunodeficiency.

Hacein-Bey-Abina S, von Kalle C, Schmidt M, Le Deist F, Wulffraat N, McIntyre E, Radford I, Villeval JL, Fraser CC, Cavazzana-Calvo M, Fischer A.

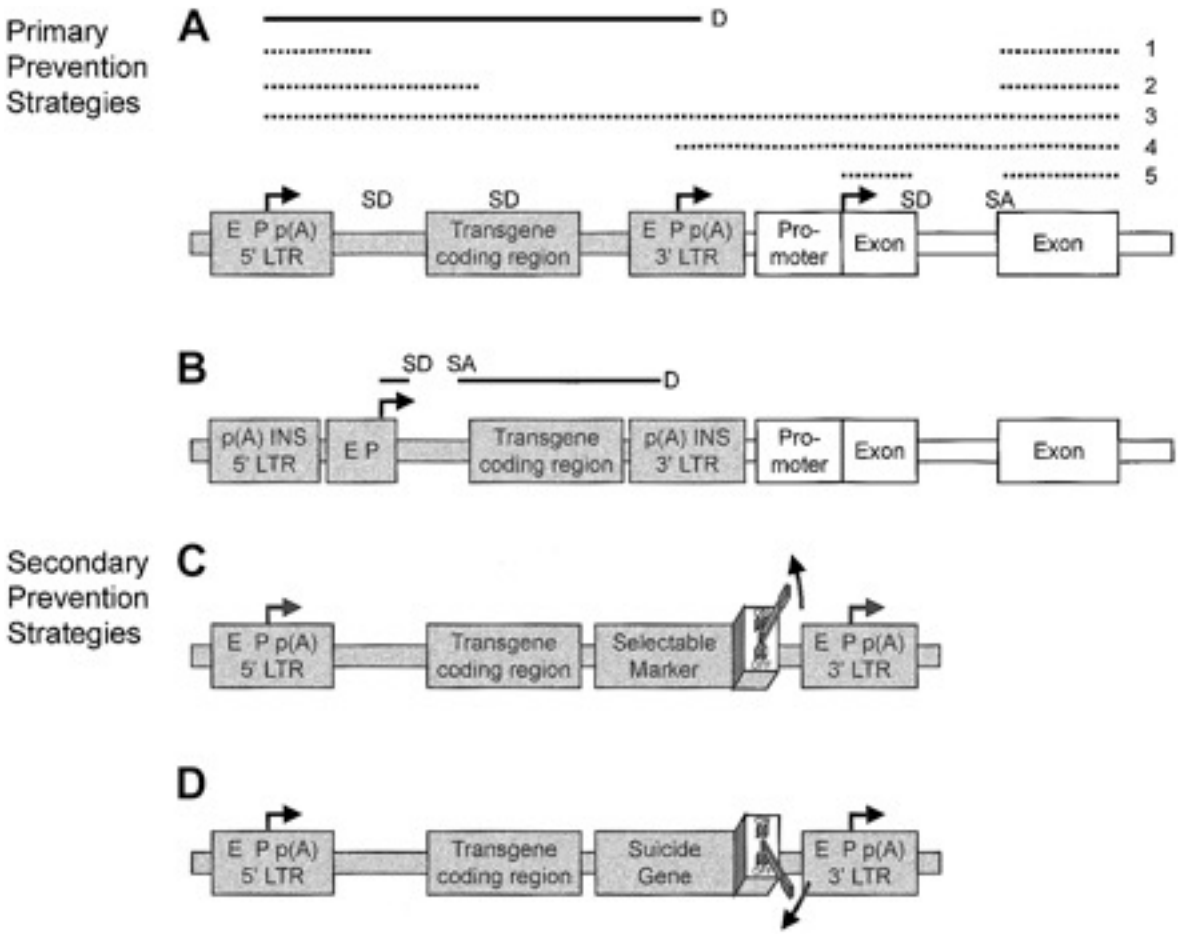
N Engl J Med 2003 Jan 16;348(3):255-6

Risks and benefits of gene therapy.

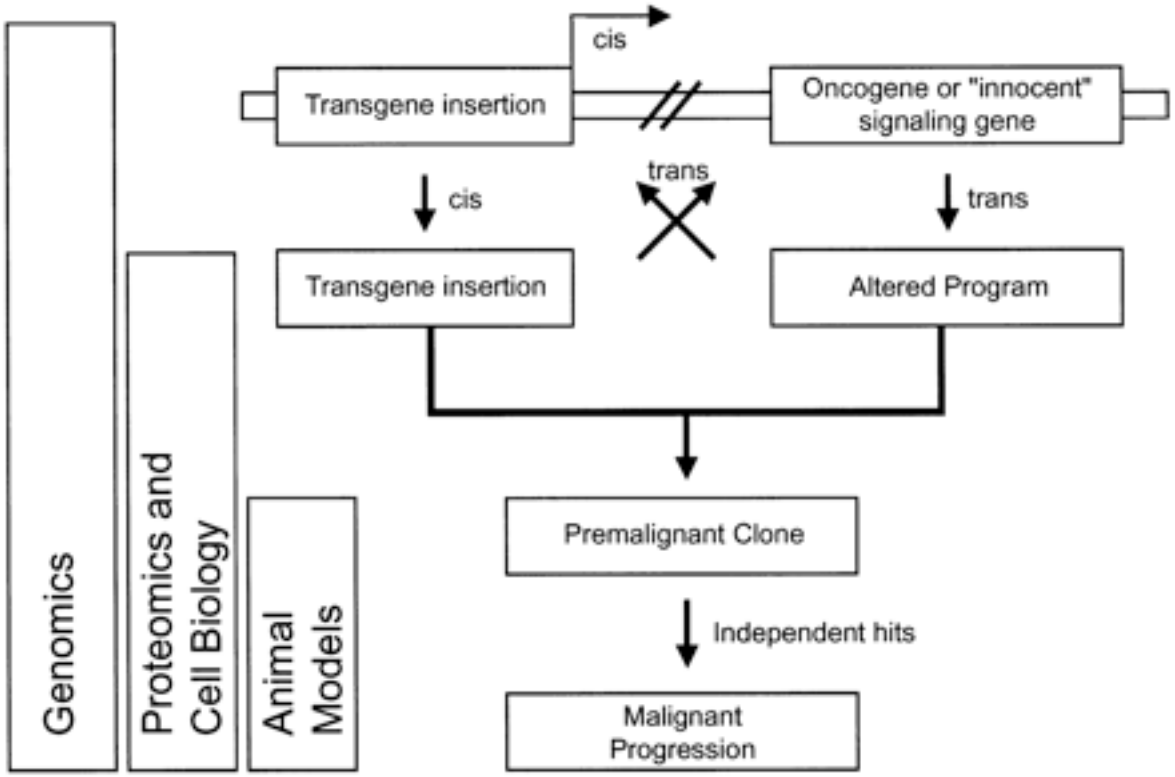
Noguchi P.

N Engl J Med 2003 Jan 16;348(3):193-4

Potenziale attivazione di geni cellulari localizzati a valle del sito di inserzione di un vettore e strategie di prevenzione



Analisi della tossicità del trasferimento genico



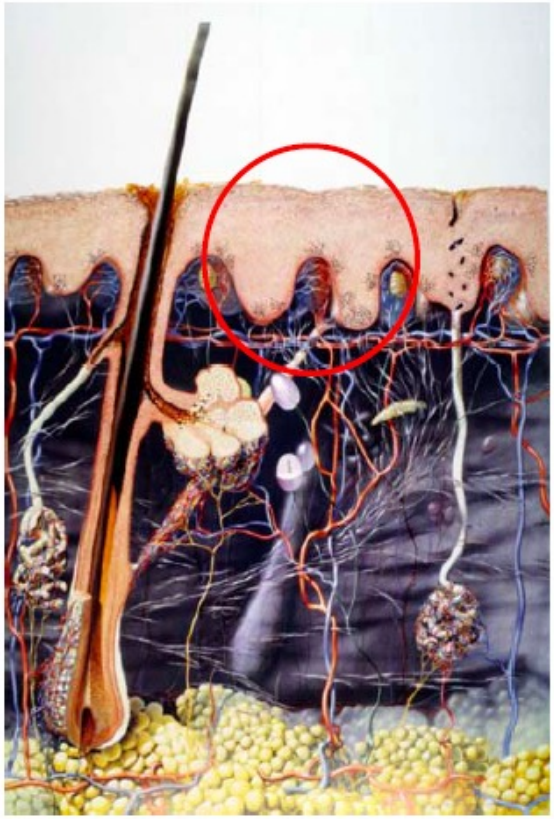
Gene therapy of genodermatosis

STRUCTURE OF HUMAN SKIN

EPIDERMIS

DERMIS

SUBCUTIS



EPITHELIAL STEM CELLS IN EPIDERMIS, HAIR FOLLICLES AND SWEAT GLANDS

MESENCHYMAL STEM CELLS IN DERMIS AND HAIR FOLLICLES

MELANOCYTE STEM CELLS

9 YEAR OLD

95% BODY AREA

THIRD DEGREE BURNS

EXCISED TO FASCIA

NO SPONTANEOUS HEALING

↓

AUTOLOGOUS SKIN TRANSPLANTATION

↓

SPLIT THICKNESS GRAFTS

and

CULTURED EPITHELIUM GRAFTS

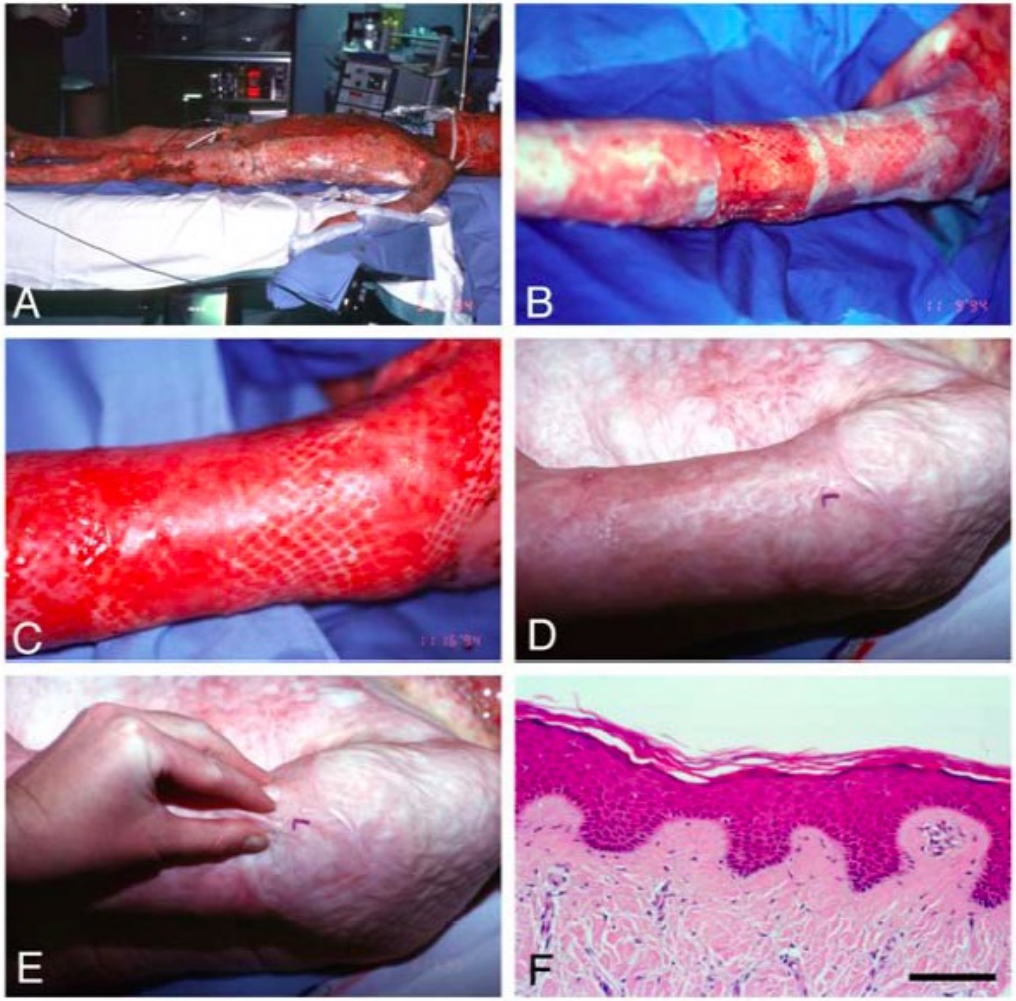
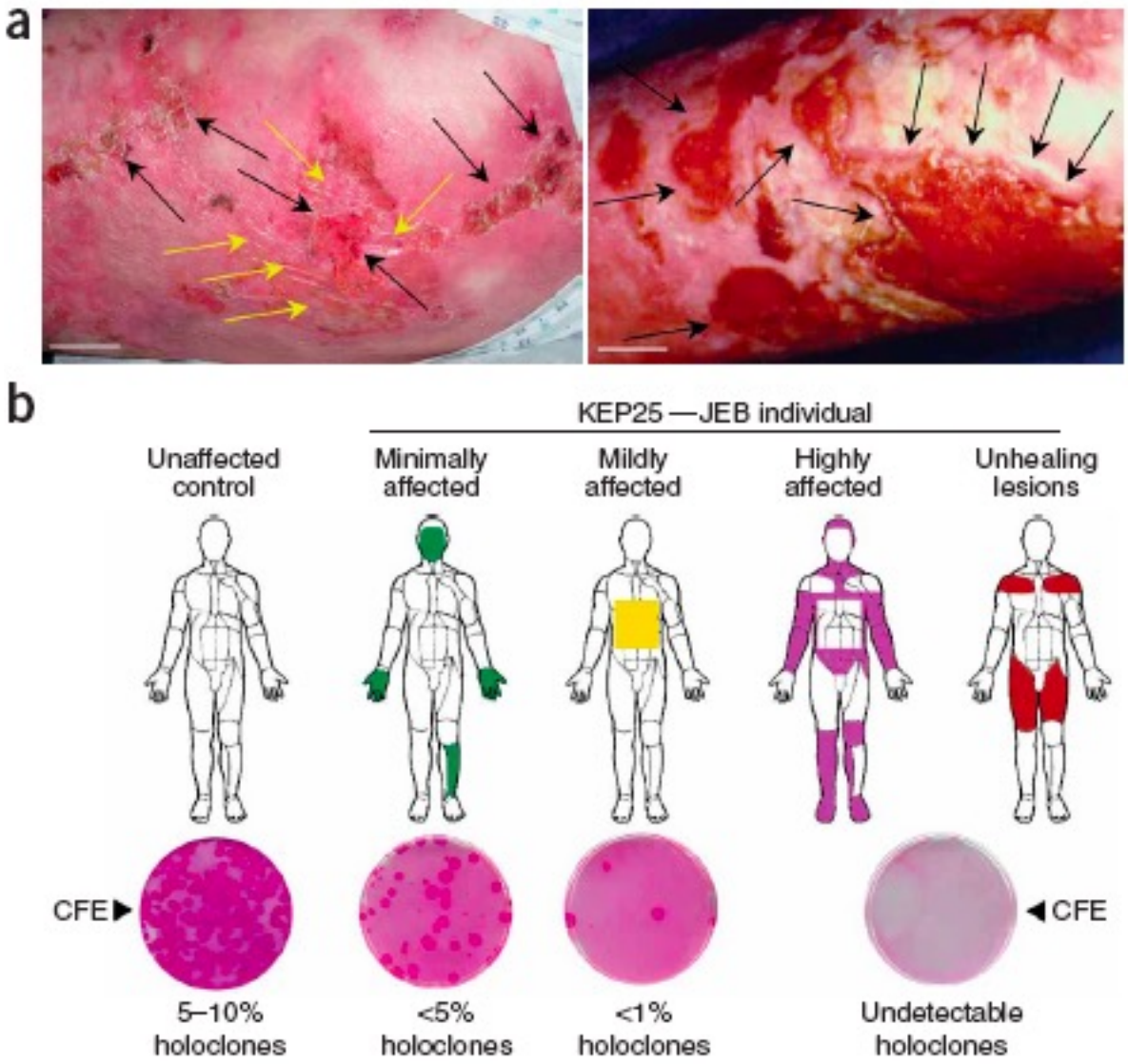


Table 1 Affected genes and proteins in different forms of epidermolysis bullosa

Disease	Affected gene	Defect
DEB	*COL7A1*	Type VII collagen
Herlitz JEB	*LAMA3*, *LAMB3*, *LAMC2*	Laminin-5 $\alpha 3$, $\beta 3$, and $\gamma 2$ chains
Benign atrophic JEB (GABEB)	*COL17A1*	Type XVII collagen (BP180)
JEB with pyloric atresia	*ITGA6*, *ITGB4*	Integrins $\alpha 6$ and $\beta 4$
EB simplex with muscular dystrophy	*PLEC1*	Plectin
EB simplex	*KRT5*, *KRT14*	Keratins 5 and 14

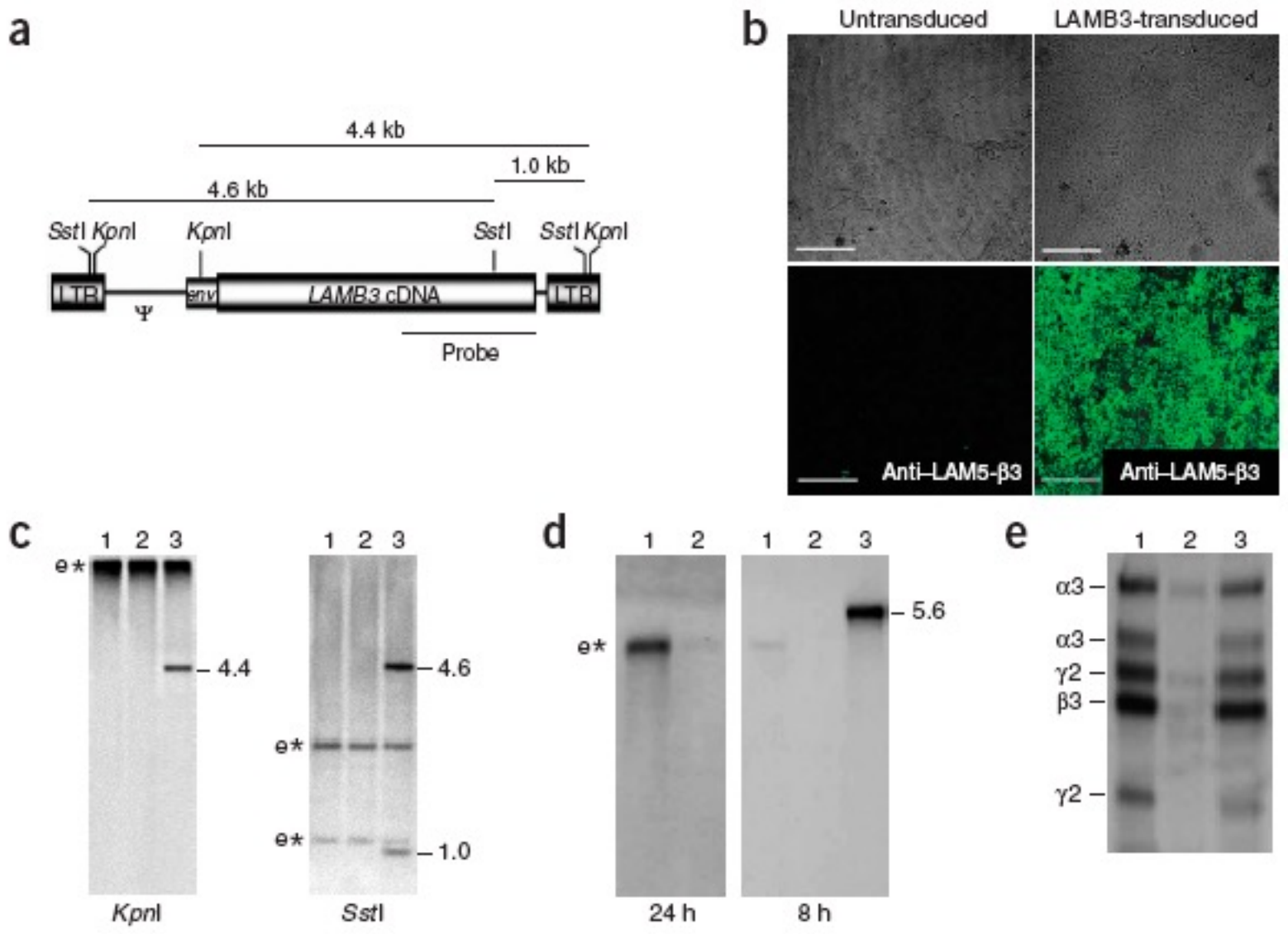
GABEB = generalized atrophic benign epidermolysis bullosa.

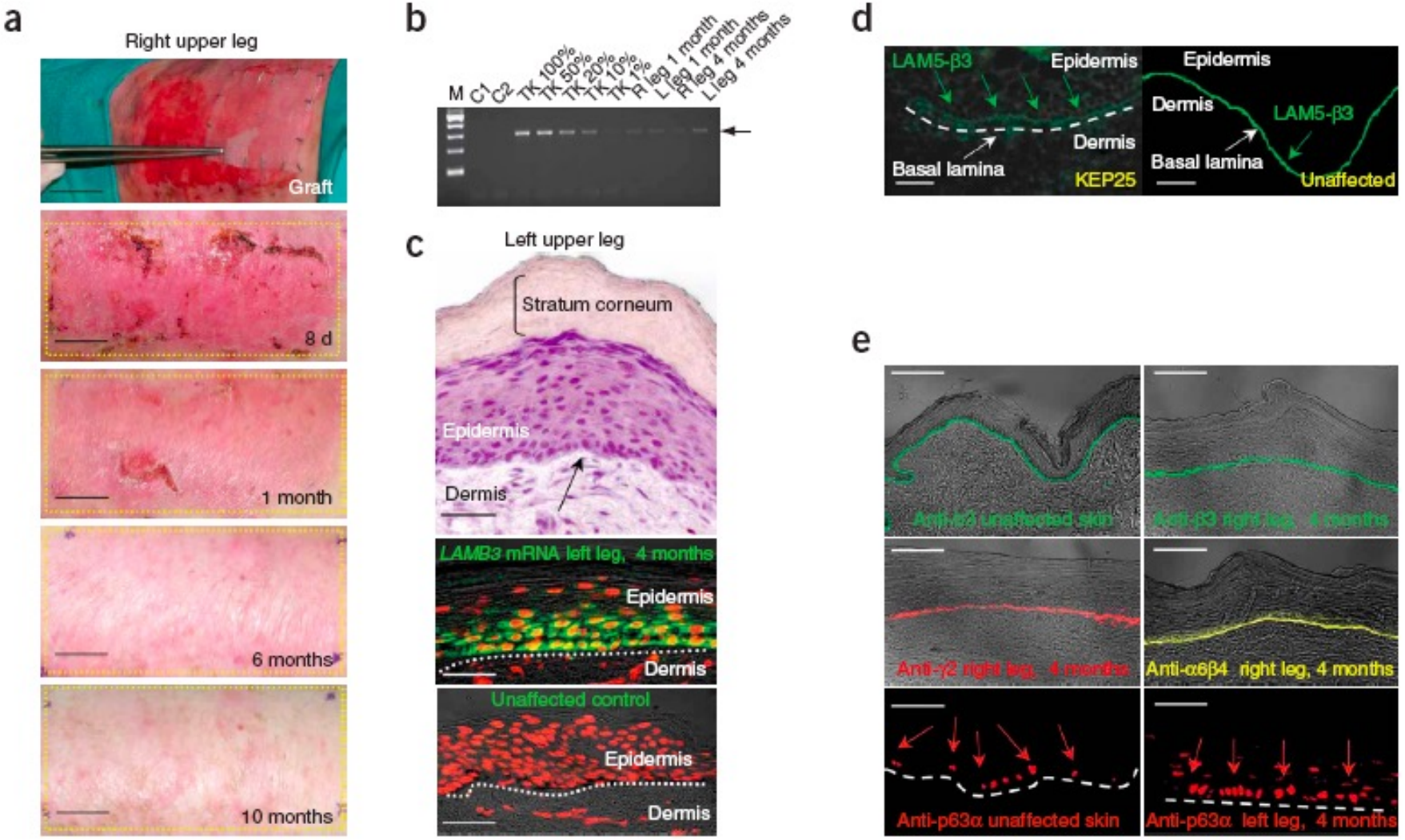

a
b
KEP25 —JEB individual
Unaffected control
Minimally affected
Mildly affected
Highly affected
Unhealing lesions
CFE
CFE
5–10% holoclones
<5% holoclones
<1% holoclones
Undetectable holoclones

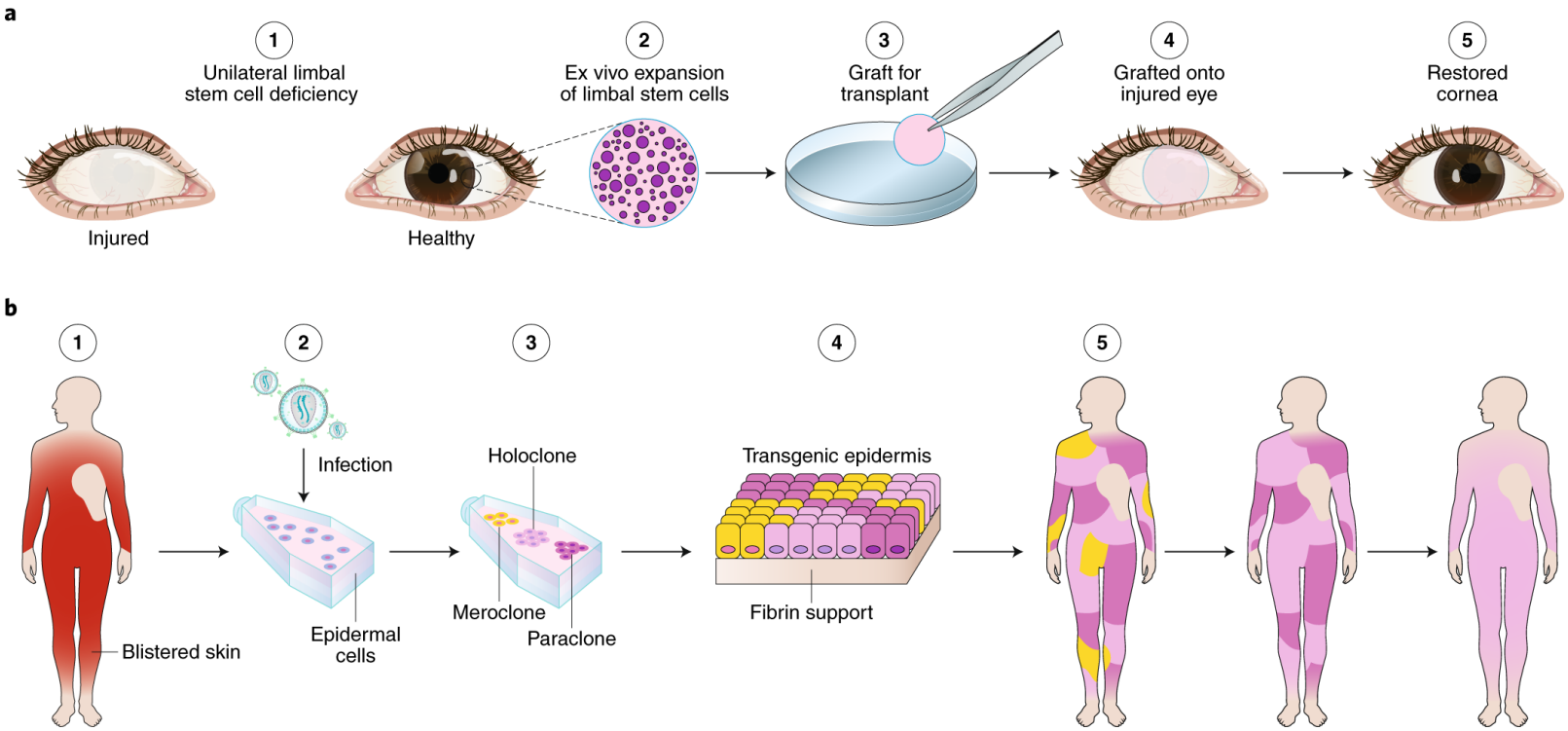
Correction of junctional epidermolysis bullosa by transplantation of genetically modified epidermal stem cells

Fulvio Mavilio[1], Graziella Pellegrini[1,2], Stefano Ferrari[2], Francesca Di Nunzio[1], Enzo Di Iorio[2], Alessandra Recchia[1], Giulietta Maruggi[1], Giuliana Ferrari[3], Elena Provasi[4], Chiara Bonini[4], Sergio Capurro[5], Andrea Conti[6], Cristina Magnoni[6], Alberto Giannetti[6] & Michele De Luca[1,2]

NATURE MEDICINE VOLUME 12 | NUMBER 12 | DECEMBER 2006


a
4.4 kb
1.0 kb
4.6 kb
SstI KpnI
KpnI
SstI
SstI KpnI
LTR
env
LAMB3 cDNA
LTR
Ψ
Probe
b
Untransduced
LAMB3-transduced
Anti–LAM5-β3
Anti–LAM5-β3
c
1 2 3
e*
4.4
1 2 3
4.6
e*
e*
1.0
KpnI
SstI
d
1 2
1 2 3
e*
5.6
24 h
8 h
e
1 2 3
α3
α3
γ2
β3
γ2

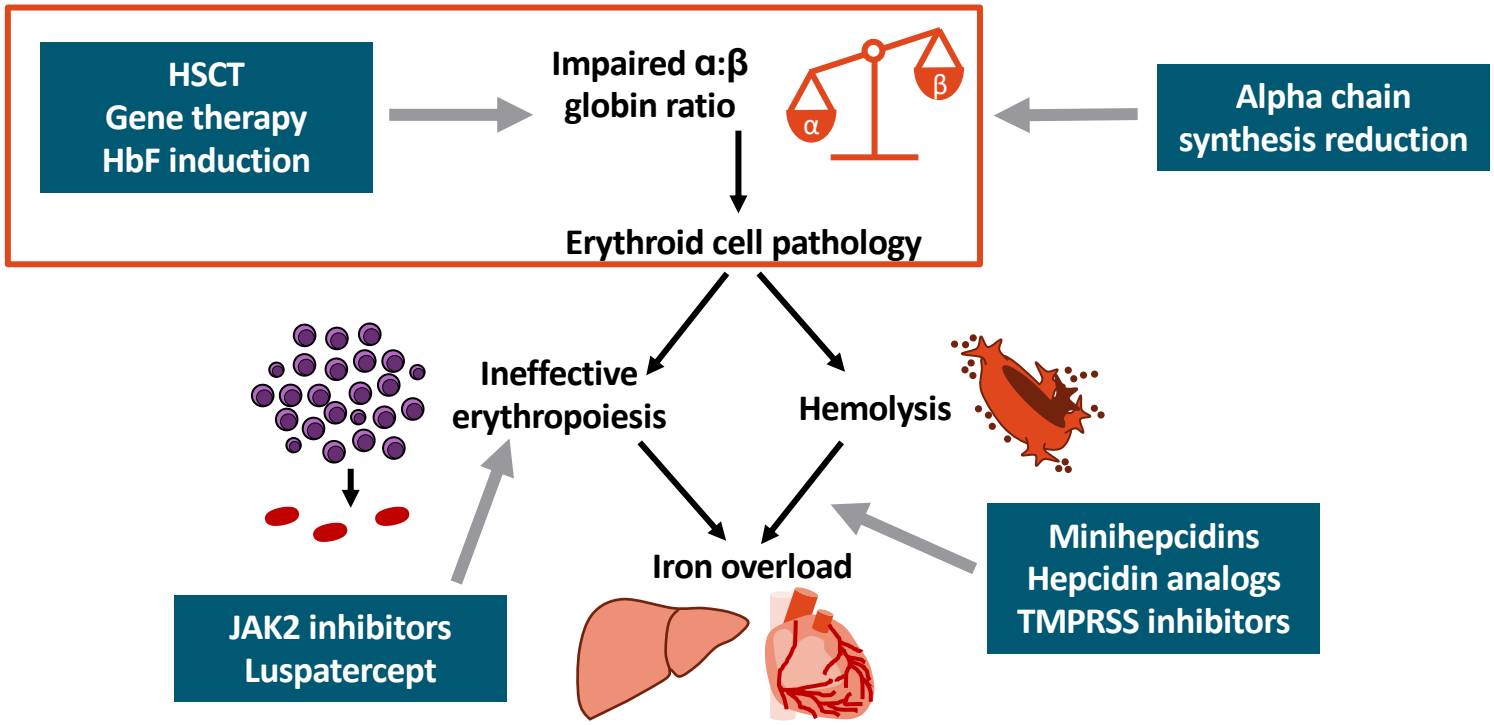

a
Right upper leg
Graft
8 d
1 month
6 months
10 months
b
M C1 C2 TK 100% TK 50% TK 20% TK 10% TK 1% R leg 1 month L leg 1 month R leg 4 months L leg 4 months
c
Left upper leg
Stratum corneum
Epidermis
Dermis
LAMB3 mRNA left leg, 4 months
Epidermis
Dermis
Unaffected control
Epidermis
Dermis
d
LAM5-β3
Epidermis
Dermis
Basal lamina
KEP25
Epidermis
Dermis
LAM5-β3
Basal lamina
Unaffected
e
Anti-α3 unaffected skin
Anti-β3 right leg, 4 months
Anti-γ2 right leg, 4 months
Anti-α6β4 right leg, 4 months
Anti-p63α unaffected skin
Anti-p63α left leg, 4 months


a
1
Unilateral limbal stem cell deficiency
2
Ex vivo expansion of limbal stem cells
3
Graft for transplant
4
Grafted onto injured eye
5
Restored cornea
Injured
Healthy
b
1
2
3
4
5
Infection
Holoclone
Transgenic epidermis
Blistered skin
Epidermal cells
Meroclone
Paraclone
Fibrin support

CLINICAL CARE OPTIONS®
ONCOLOGY

Gene Therapy for β-Thalassemia

Emerging Therapies for Thalassemias



Cappellini. Hematology Am Soc Hematol Educ Program. 2017;2017:278.

Slide credit: clinicaloptions.com

Hematopoietic Stem Cell Transplantation (HSCT) vs Gene Therapy

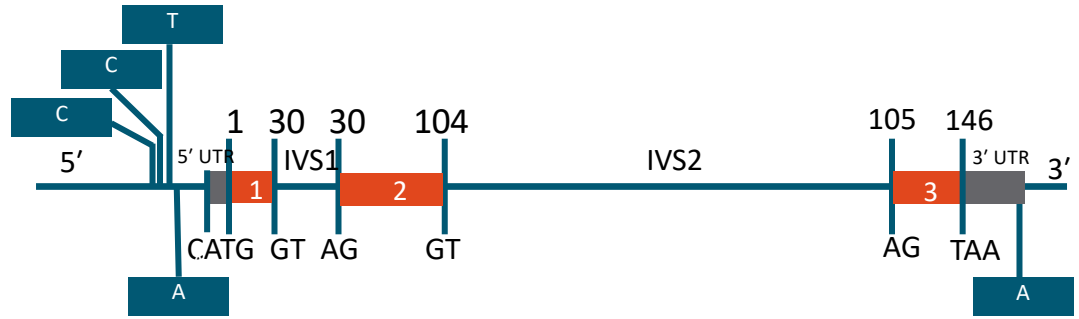
Parameter	HSCT	Gene Therapy
Chemotherapy	✓	✓
Immunosuppression	✓	None
Graft-vs-host disease	✓	None
Donor availability	Allogenic – limited	Autologous – available
Insertional mutation	None	Possible

Slide credit: clinicaloptions.com

Mutations in the β-Globin Gene Causing β-Thalassemia

β-globin gene

β-thalassemia
> 200 mutations



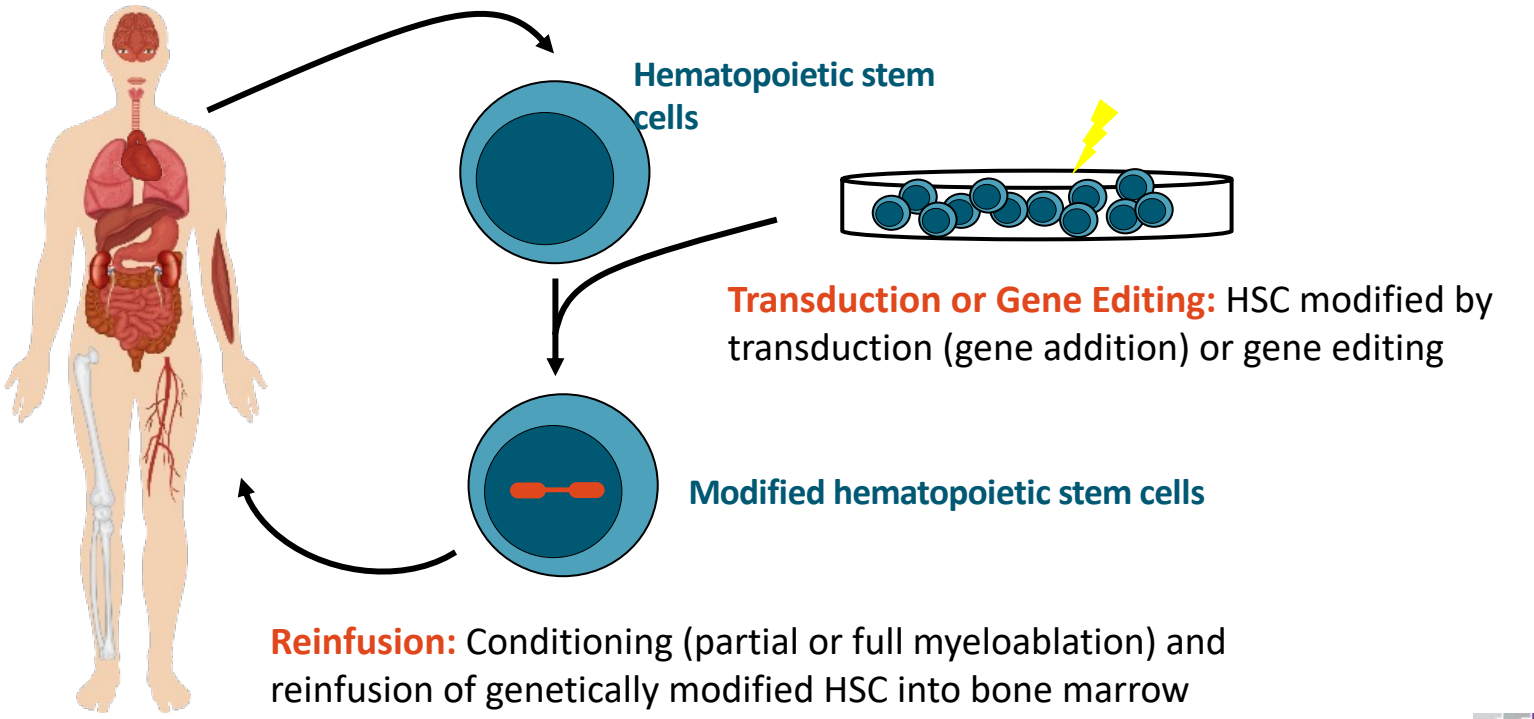
- β^0: mutated gene cannot produce any β-globin
- β^+: mutated gene produces reduced amount of β-globin

Origa. Genet Med. 2017;19:609.

Slide credit: clinicaloptions.com

Gene Therapy Approach

HSC Collection: HSCs harvested from bone marrow or by mobilization/apheresis



Rivella. Haematologica. 2015;100:418.

Slide credit: clinicaloptions.com

Gene Therapy for β-Hemoglobinopathies: Requirements

- High levels of expression, especially for severe phenotypes
- Controlled, erythroid-specific gene expression
- Stable, long-term production of gene product
- No insertional mutagenesis or off-target effects

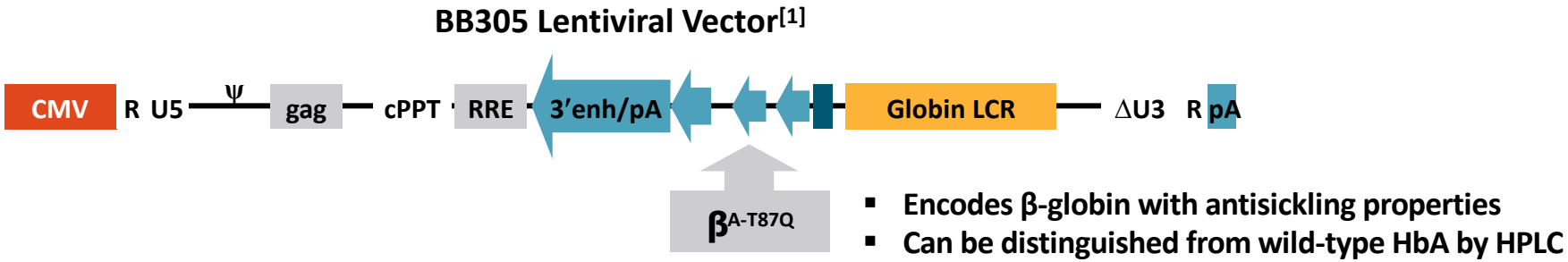
Slide credit: clinicaloptions.com

Gene Therapy for β-Hemoglobinopathies: Approaches

- **Globin gene addition**
  - **Functional β-globin gene**
  - Functional γ-globin gene
- Gene editing
  - Reverse fetal hemoglobin repression
    - BCL11A
  - Correct the β-globin mutation

Slide credit: clinicaloptions.com

LentiGlobin BB305 Vector–Based Gene Therapy in Transfusion-Dependent β-Thalassemia



- LentiGlobin BB305 vector encodes adult hemoglobin (HbA) with a T87Q amino acid substitution (HbA^{T87Q})[2]
- Phase I/II HGB-204 and HGB-205 studies of 22 patients (12-35 yrs of age) treated with transfusion-dependent β-thalassemia[2]
- Assessments included AEs, vector integration, levels of replication-competent virus, levels of total Hb and HbA^{T87Q}, transfusion requirements, and average VCN[2]

1. Negre. Hum Gene Ther. 2016;27:148. 2. Thompson. NEJM. 2018;378:1479.

Slide credit: clinicaloptions.com

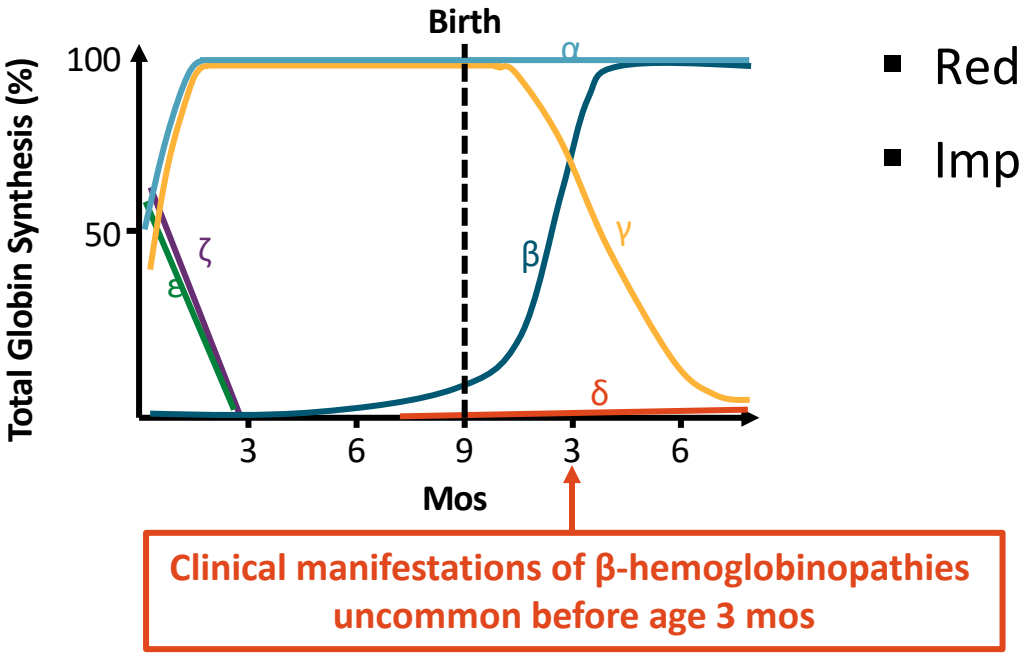
Gene Therapy for β-Hemoglobinopathies: Approaches

- Globin gene addition
  - Functional β-globin gene
  - Functional γ-globin gene
- **Gene editing**
  - **Reverse fetal hemoglobin repression**
    - **BCL11A**
  - Correct the β-globin mutation

Rivella. Haematologica. 2015;100:418.

Slide credit: clinicaloptions.com

Induction of Fetal Hemoglobin: Rationale

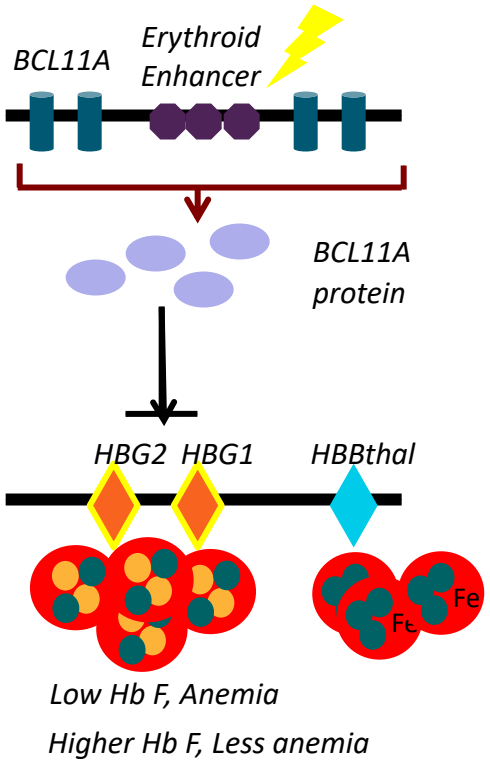


- Improve α- to β-like ratio
- Reduce ineffective erythropoiesis
- Improve anemia

Slide credit: clinicaloptions.com

Induction of Fetal Hemoglobin: BCL11A as Possible Target

- BCL11A, an erythroid enhancer, represses fetal hemoglobin production
- Provides potential erythroid-specific targeting
  - Avoid off-target effects (lymphocytes, HSC)



Bauer. Science. 2013;342:253.

Slide credit: clinicaloptions.com

Terapia genica delle malattie degenerative

Distrofia muscolare

Panel 1: **Gene loci and protein defects in the commonest forms of muscular dystrophy**

Disorder	Gene locus	Protein defect
Congenital (AR)	6q	Laminin α2 (merosin)
	12q	Laminin receptor (α7 integrin)
	9q	Fukutin (Fukuyama dystrophy)
	1p	Selenoprotein N1 (rigid spine syndrome)
	1p	Glycosyltransferase (muscle-eye-brain disease)
Duchenne and Becker (XR)	Xp21	Dystrophin
Emery-Dreifuss (XR)	Xq28	Emerin
Emery-Dreifuss (AD/AR)	1q	Lamin A/C
Distal (AD)	14q, 2q	?
Distal (AR)	2p	Dysferlin
Facioscapulohumeral (AD)	4q	?
Oculopharyngeal (AD)	14q	Poly(A)-binding protein 2 (PAB 2)
Limb-girdle (AD)		
1A	5q	Myotilin
1B	1q	Lamin A/C
1C	3p	Caveolin 3
1D	6q	?
1E	7q	?
1F	2q	?
Limb-girdle (AR)		
2A	15q	Calpain-3
2B	2p	Dysferlin
2C	13q	γ-sarcoglycan
2D	17q	α-sarcoglycan (adhalin)
2E	4q	β-sarcoglycan
2F	5q	δ-sarcoglycan
2G	17q	Telethonin
2H	9q	?
2I	19q	Fukutin-related

Modes of inheritance: AR=autosomal recessive; AD=autosomal dominant; XR=X-linked recessive. ?=unknown.

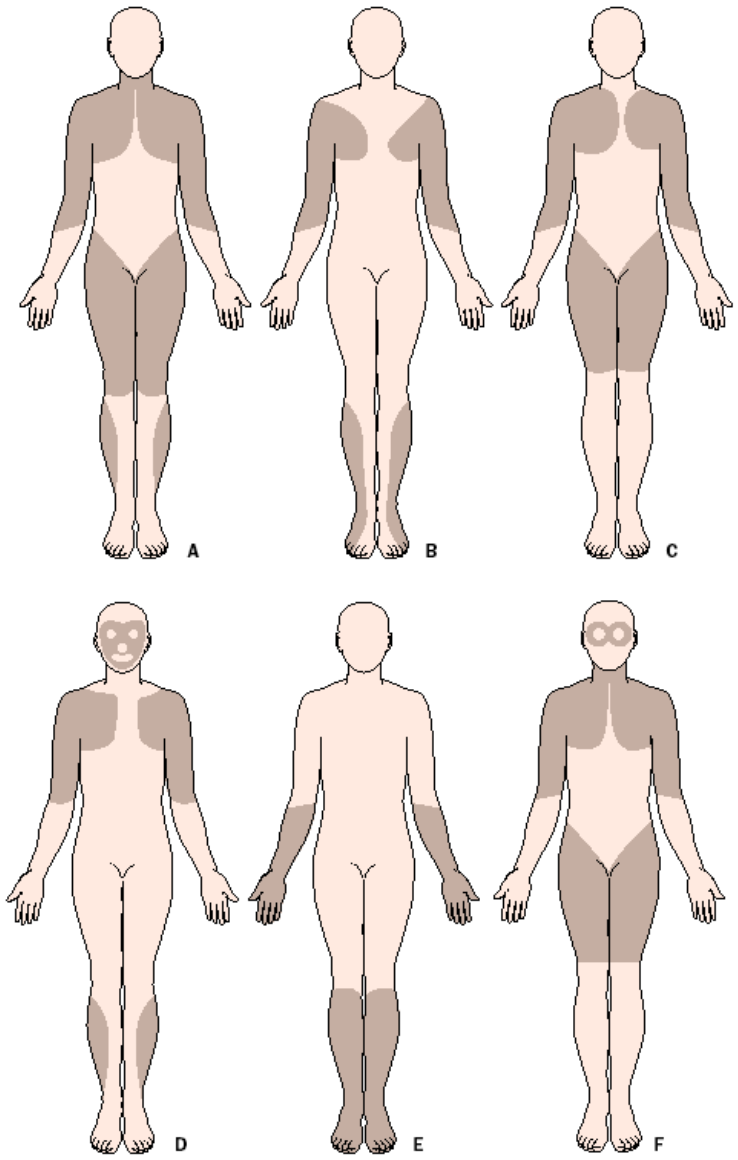
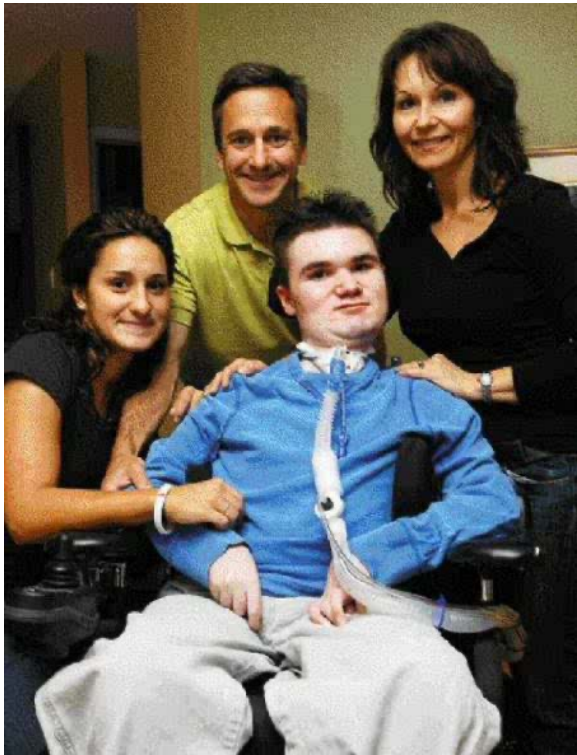


Figure 1: **Distribution of predominant muscle weakness in different types of dystrophy** A, Duchenne-type and Becker-type; B, Emery-Dreifuss; C, limb-girdle; D, facioscapulohumeral; E, distal, F, oculopharyngeal. Shaded=affected areas. (Reproduced from *BMJ* 1998; **317**: 991–95 by permission of the BMJ Publishing Group).

Duchenne Muscular Dystrophy

the **most common of several childhood muscular dystrophies**, it is an **inherited** disorder (**X-linked recessive**) with progressive **degeneration of muscle**, **onset** is generally **before age 6 years**

People with DMD **lose muscle all there lives**, but it is **usually not noticed** until a parent or caretaker finds **unusual walking and/or talking around the age of 3**

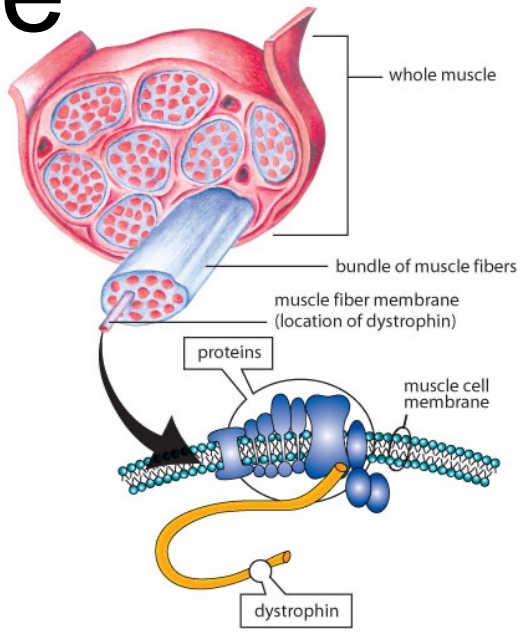


Incidence Rates

Although **girls rarely get this disease**, females can still have some of the symptoms like **weaker muscles in the back, legs and arms that fatigue easily**. Some may need a wheelchair or other mobility aids**. Carriers may have heart problems, and can have shortness of breath or failure to do moderate exercise.** The heart problems, if untreated, **can be quite serious, even life-threatening**

About** 1 in every 3,500 **boys** is **born** with **DMD

Cause

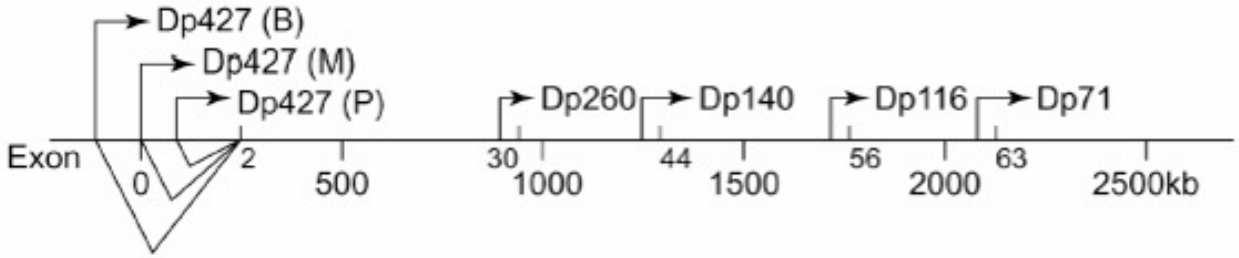


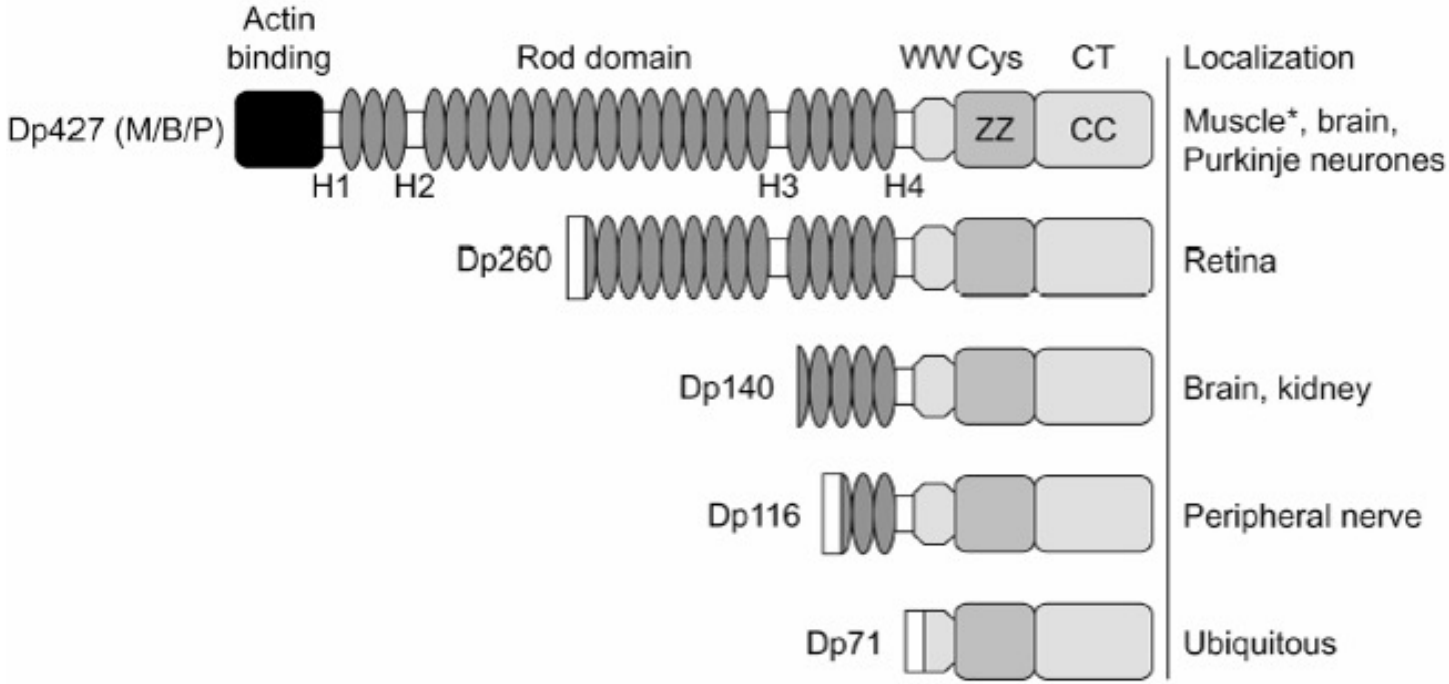
DMD is **caused by a mutation in the gene** that **produces an important muscle protein called dystrophin**, which is not produced

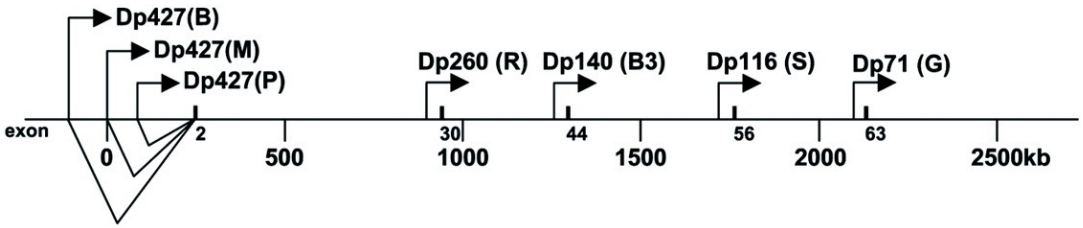
The Muscle-Fiber Membrane

Muscles are made up of bundles of fibers (cells). A group of interdependent proteins along the membrane surrounding each fiber helps to keep muscle cells working properly. When one of these proteins, dystrophin, is absent, the result is Duchenne muscular dystrophy.

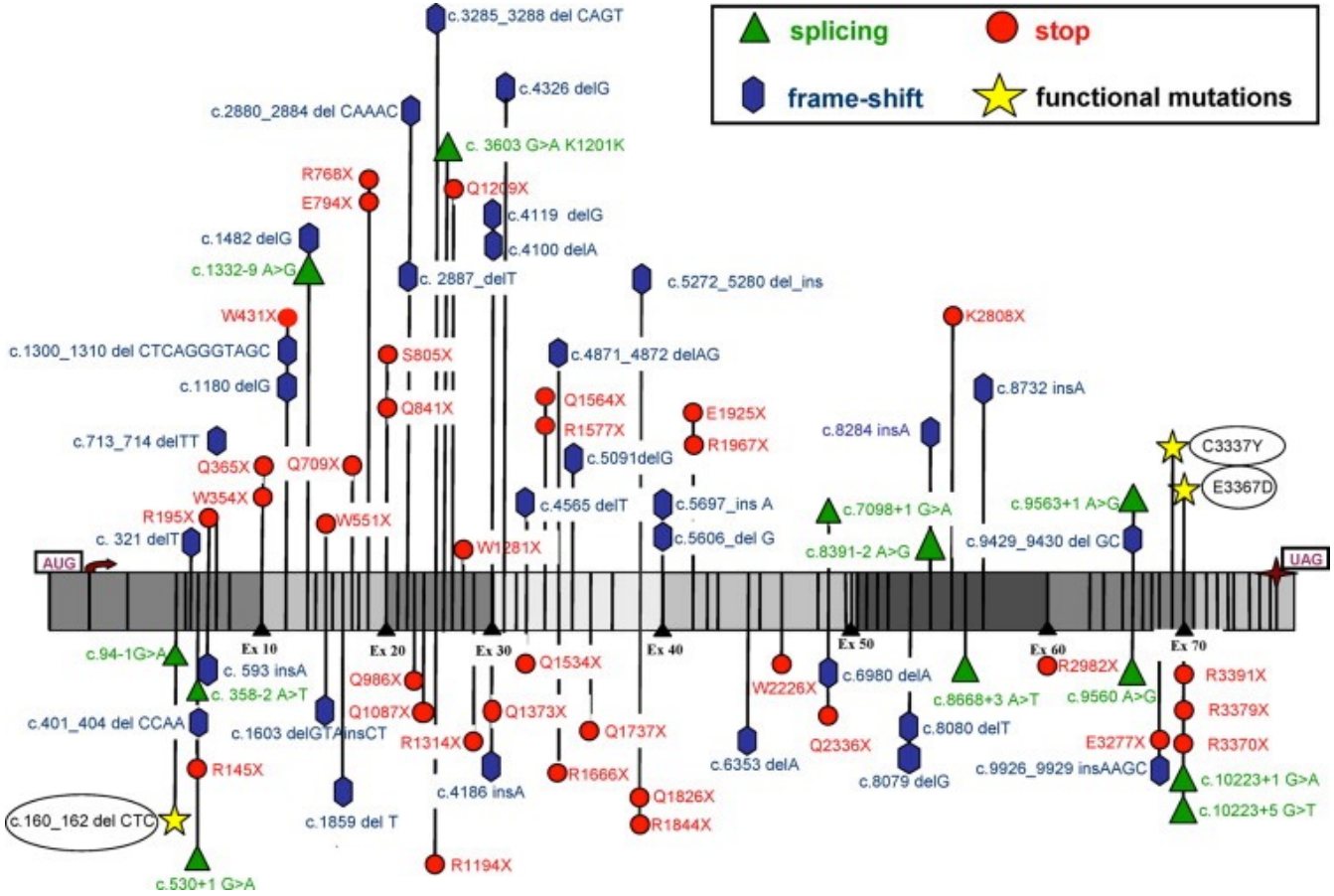
Dystrophin Gene



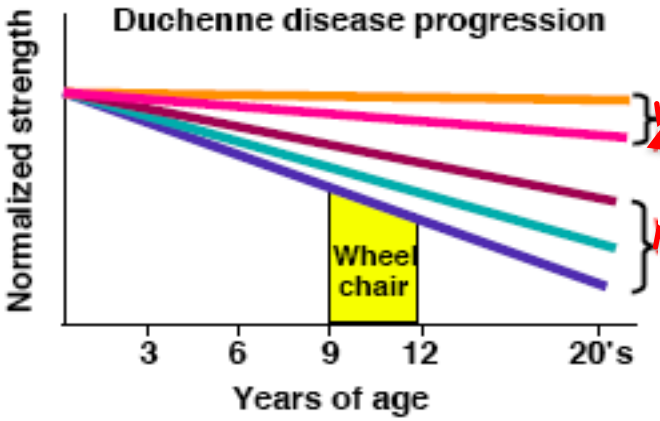



Dp427(B)
Dp427(M)
Dp427(P)
Dp260 (R)
Dp140 (B3)
Dp116 (S)
Dp71 (G)
exon
0
2
30
44
56
63
500
1000
1500
2000
2500kb


ACTIN-
BINDING
ROD DOMAIN
WW
CYS
CT
DYSTROPHIN
1
2
3
4
EF/ZZ
CC


splicing
stop
frame-shift
functional mutations
c.3285_3288 del CAGT
c.4326 delG
c.2880_2884 del CAAAC
c. 3603 G>A K1201K
R768X
E794X
Q1209X
c.4119 delG
c.4100 delA
c.1482 delG
c.1332-9 A>G
c. 2887_delT
c.5272_5280 del_ins
W431X
K2808X
c.1300_1310 del CTCAGGGTAGC
S805X
c.4871_4872 delAG
c.8732 insA
c.1180 delG
Q841X
Q1564X
R1577X
E1925X
R1967X
c.8284 insA
c.713_714 delTT
c.5091delG
C3337Y
E3367D
Q365X
Q709X
W354X
c.4565 delT
c.5697_ins A
c.7098+1 G>A
c.9563+1 A>G
R195X
W551X
c.5606_del G
c.8391-2 A>G
c.9429_9430 del GC
c. 321 delT
W1281X
AUG
UAG
Ex 10
Ex 20
Ex 30
Ex 40
Ex 50
Ex 60
Ex 70
c.94-1G>A
c. 593 insA
Q1534X
W2226X
c.6980 delA
R2982X
R3391X
Q986X
c. 358-2 A>T
c.8668+3 A>T
c.9560 A>G
R3379X
c.401_404 del CCAA
Q1087X
Q1373X
c.1603 delGTAinsCT
Q1737X
c.8080 delT
E3277X
R3370X
R1314X
Q2336X
c.6353 delA
R145X
R1666X
c.9926_9929 insAAGC
c.10223+1 G>A
c.4186 insA
c.8079 delG
Q1826X
c.160_162 del CTC
c.1859 del T
R1844X
c.10223+5 G>T
R1194X
c.530+1 G>A

Strategie di trattamento e cura per la DMD e altre Distrofie: situazione attuale e prospettive



Terapia Genica:

Il gene della distrofina è molto lungo (25000 bp) , non è ancora tecnicamente possibile trasferirlo tutto

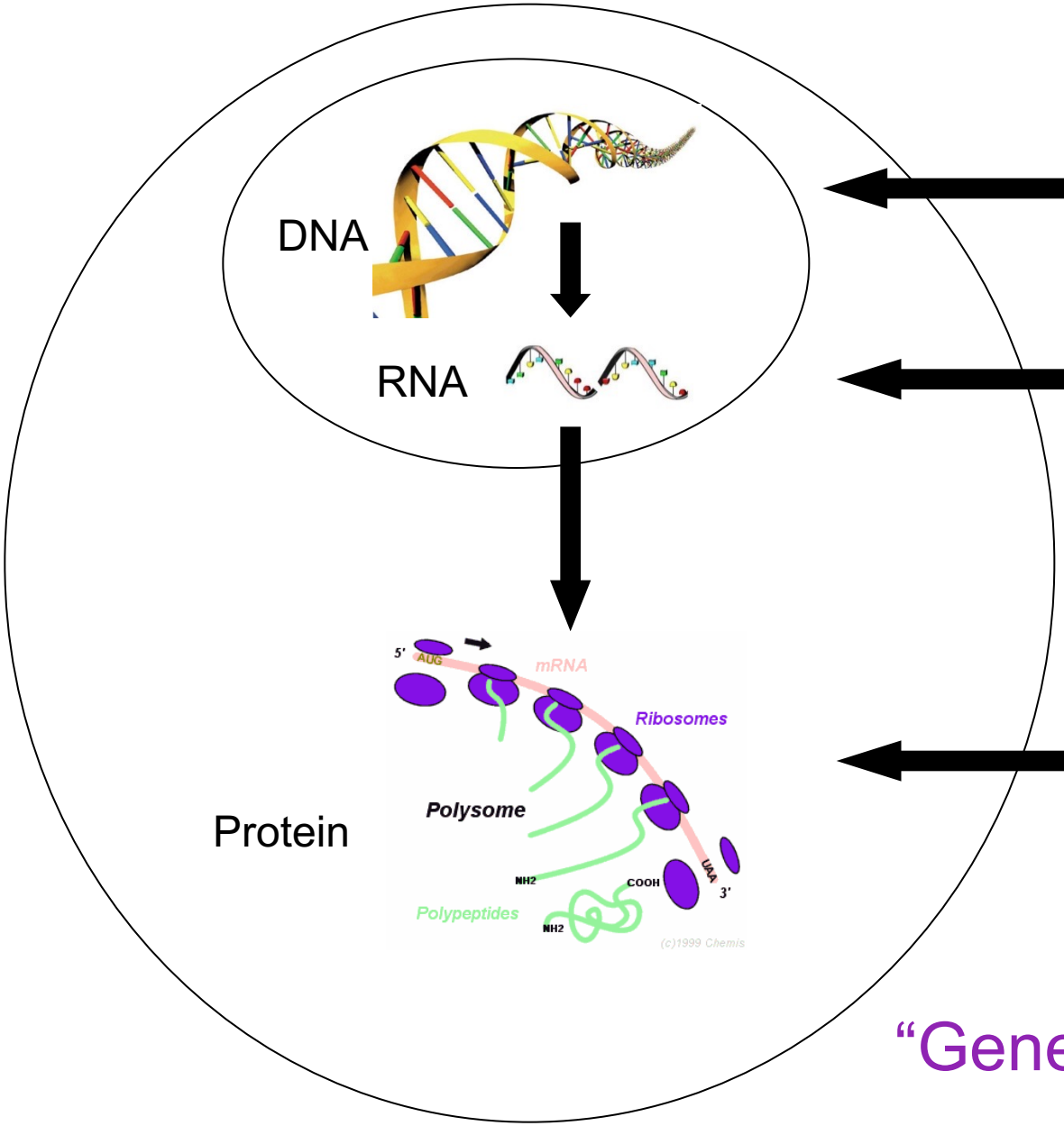
Terapia Cellulare:

Quali cellule staminali?

Strategie Farmacologiche

- Gluococorticoidi (steroidi)
- Fattori di crescita (IGF1)
- Inibitori TNF-α
- Ossido Nitrico
- Inibitori della miostatina

Tutti questi trattamenti sono palliativi
ritardano di poco il peggioramento della patologia



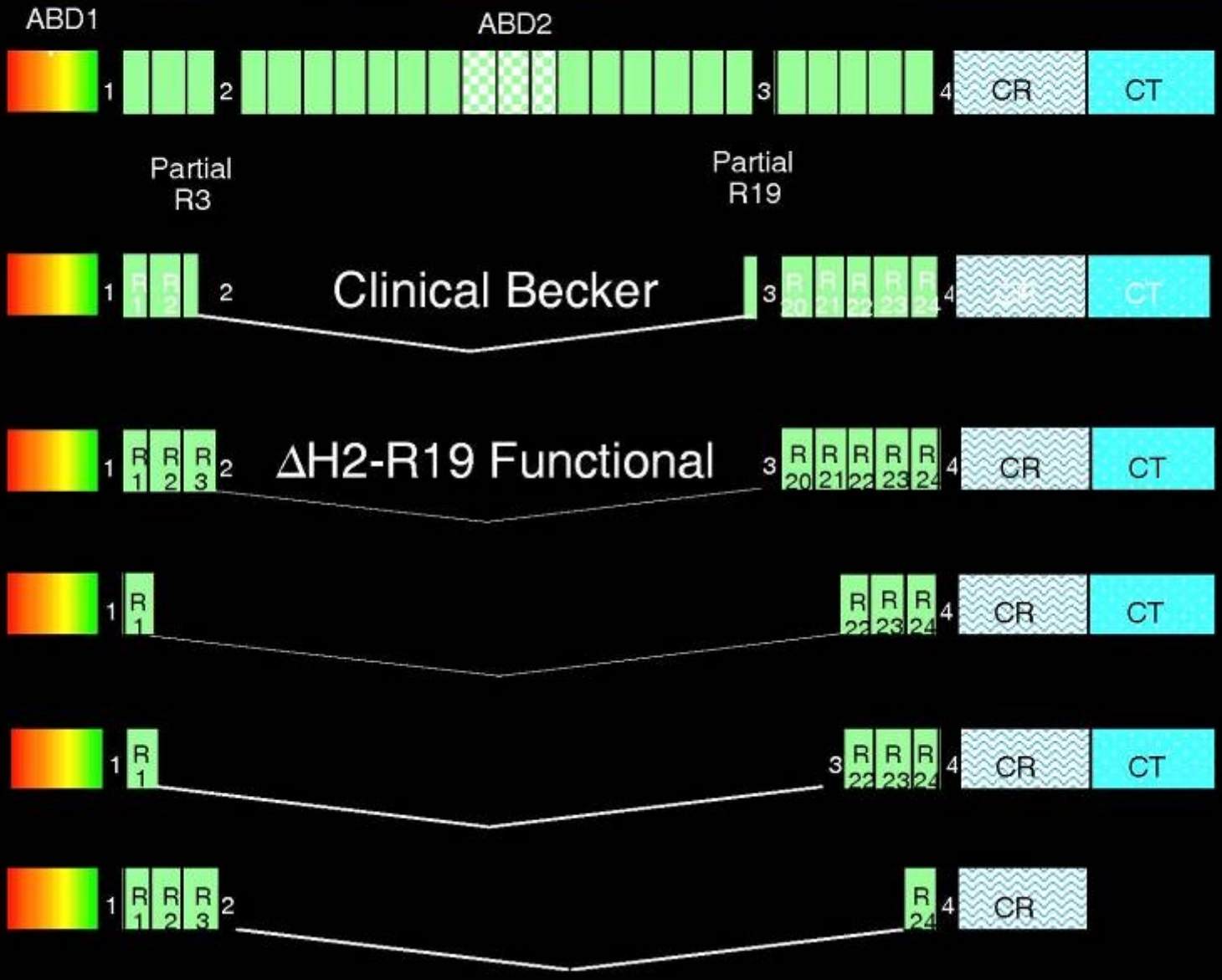
Gene Addition
e.g. microdystrophin
via viral vector.
Cell therapy.

Modify RNA
e.g. exon-skipping

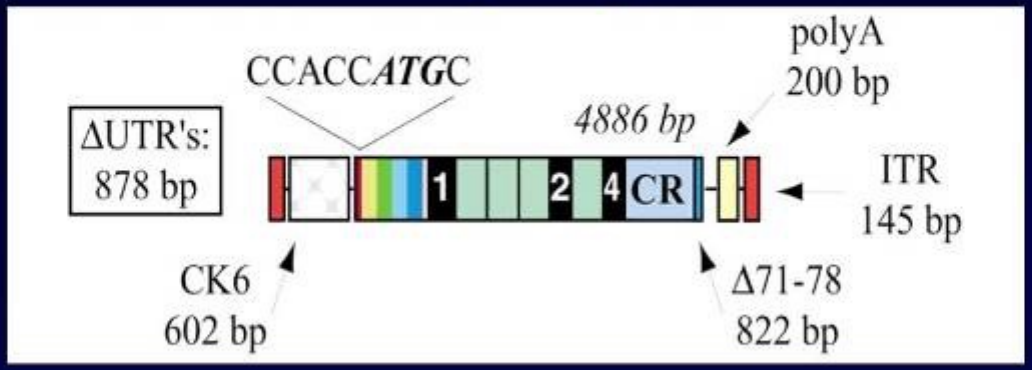
Modify translation
e.g. gentamycin
PTC 124 (ataluren)

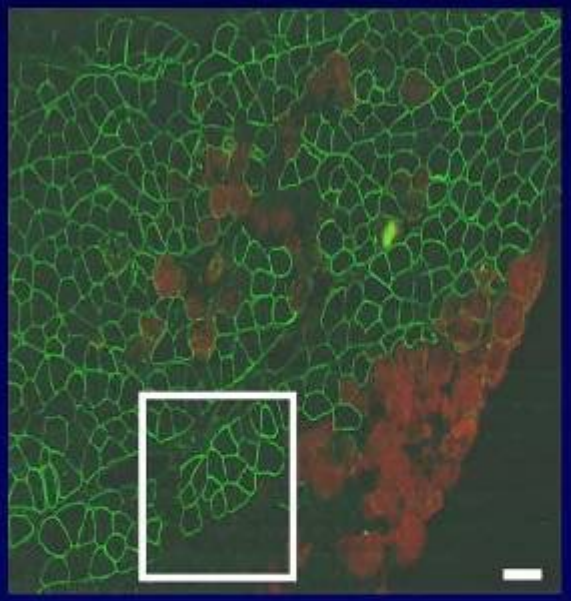
"Genetic" therapies

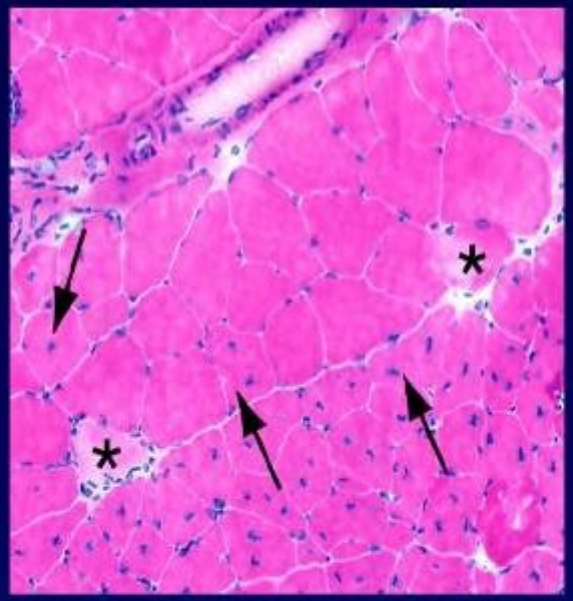
Mini- and micro-dystrophins

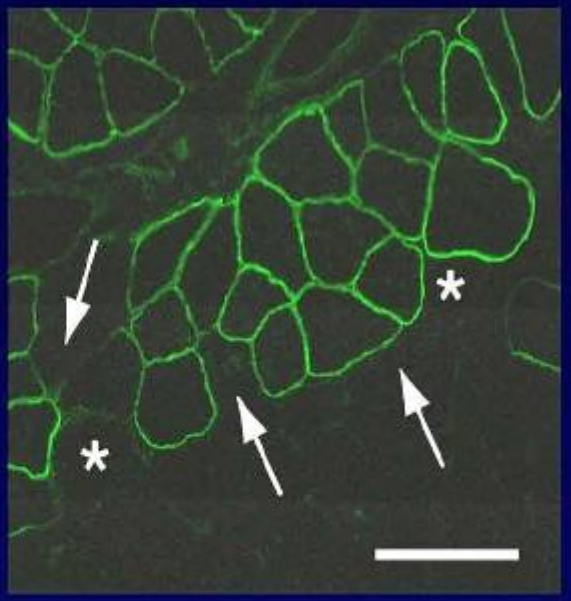


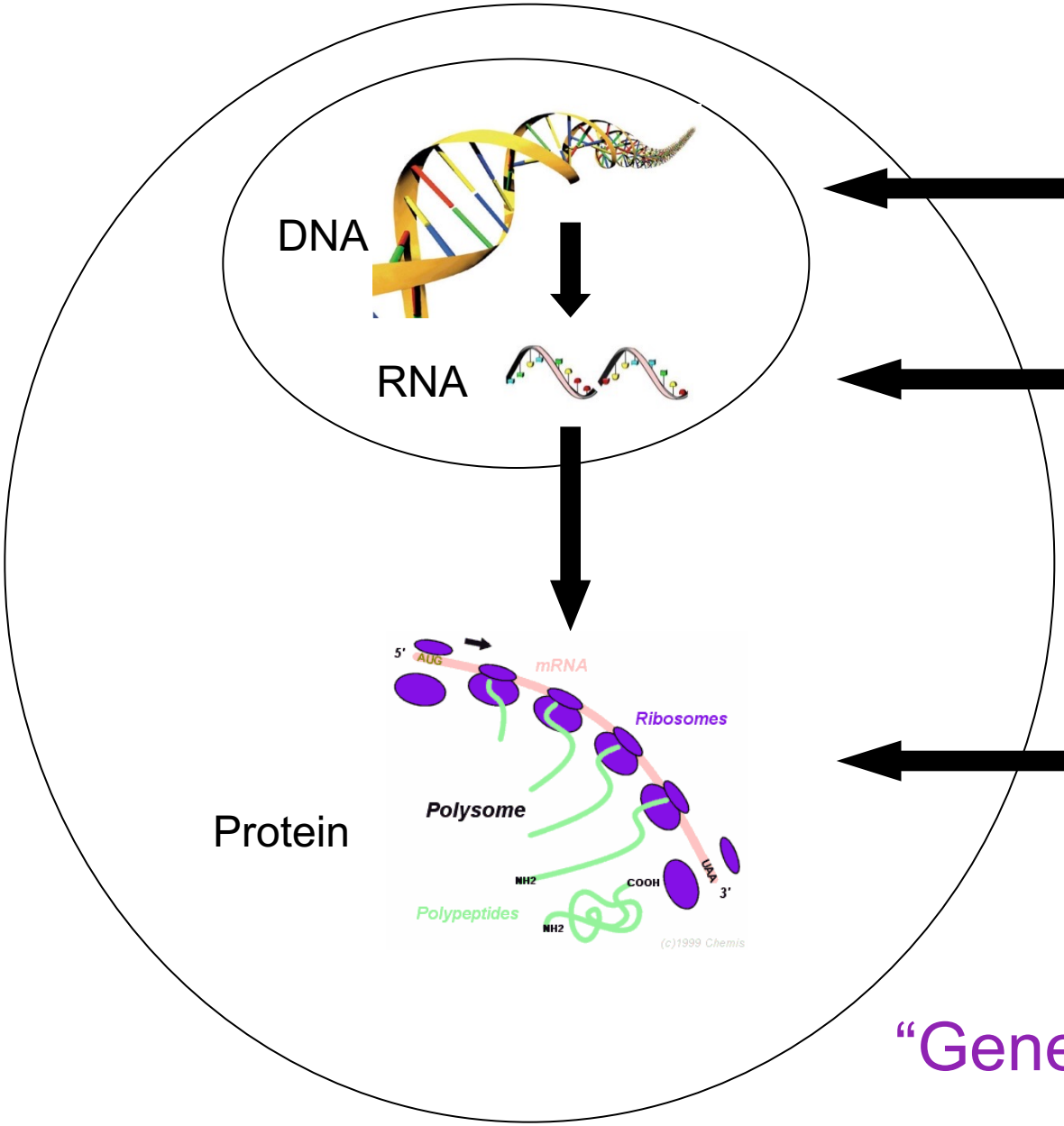
Analysis of Mouse Muscle Injected With AAV•Micro-Dystrophin-5 months later











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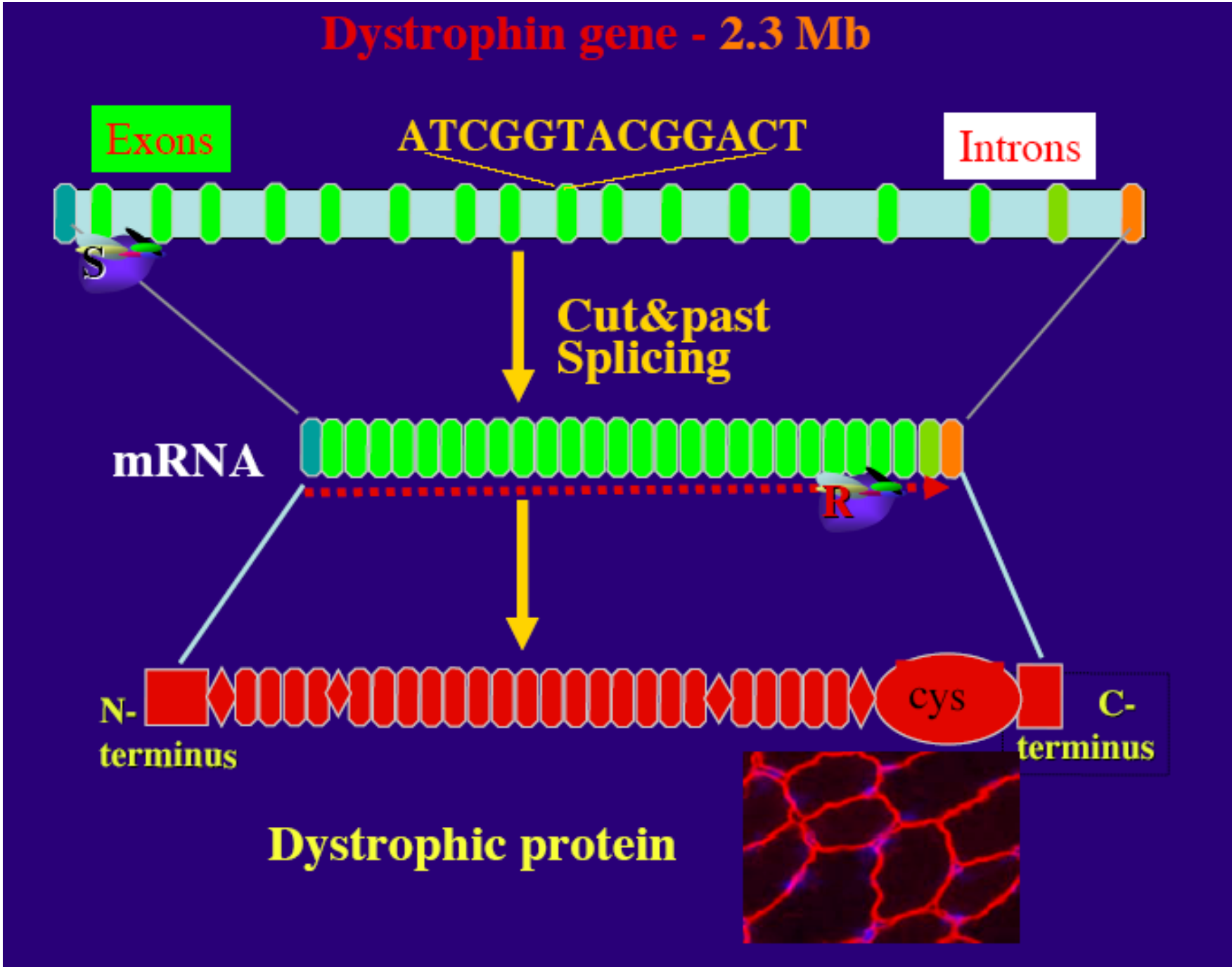
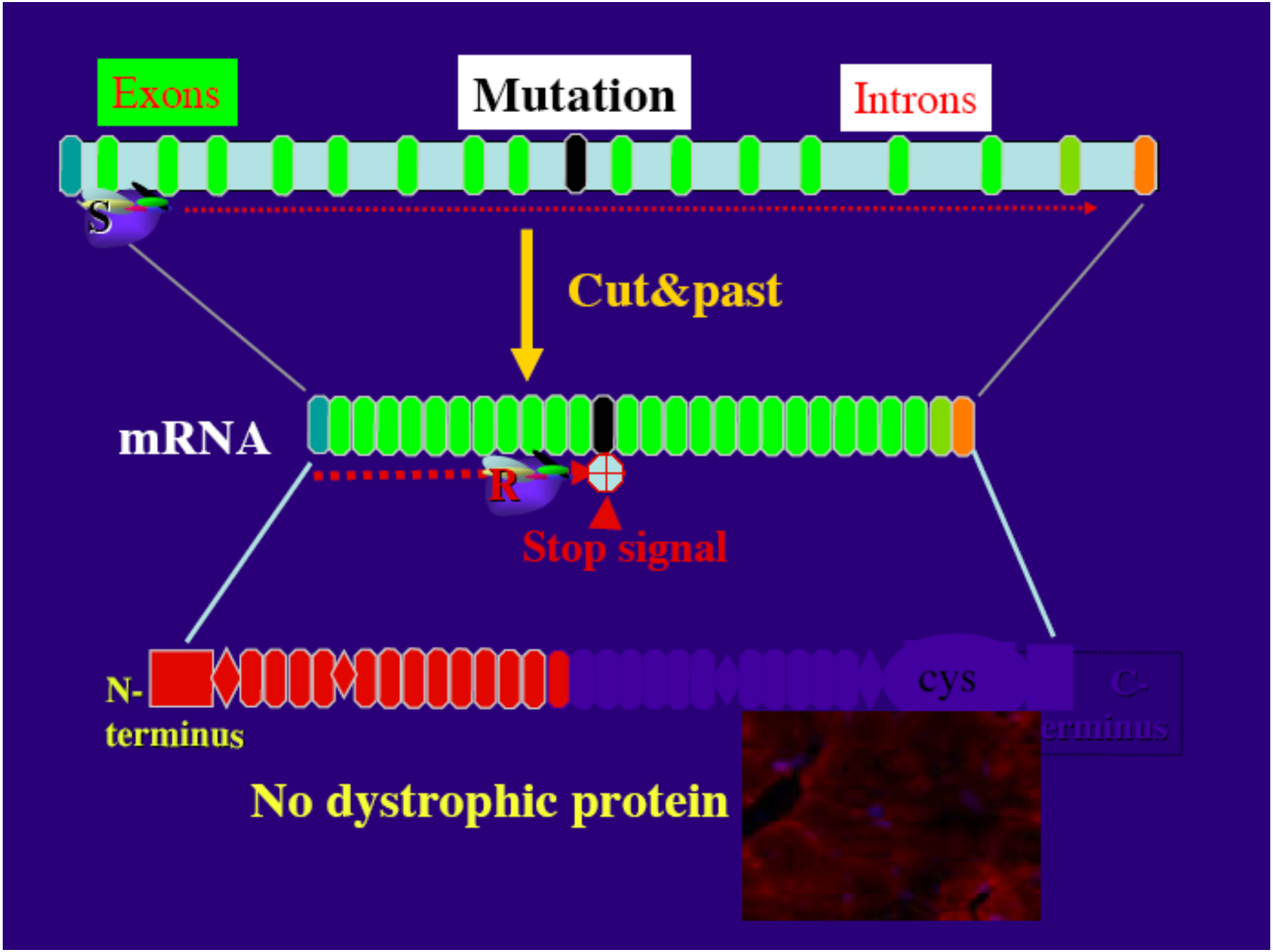
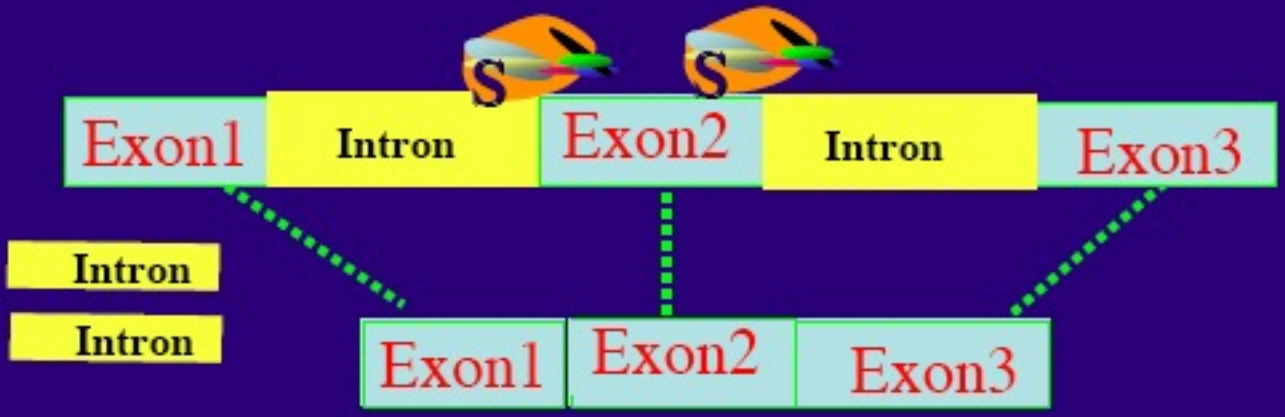
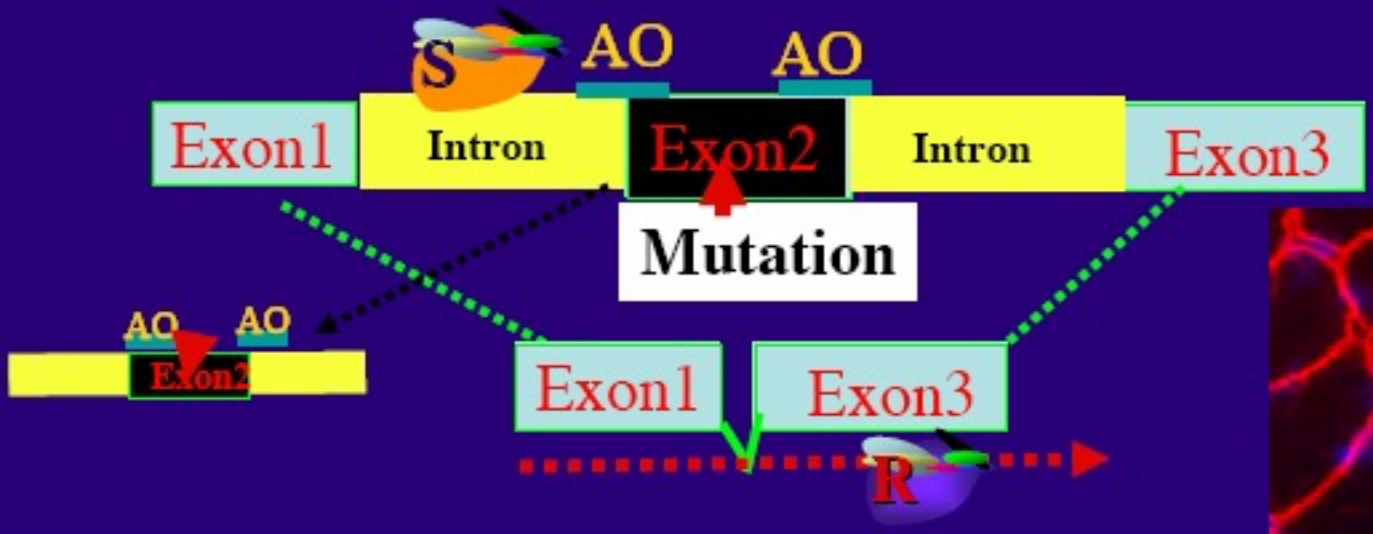
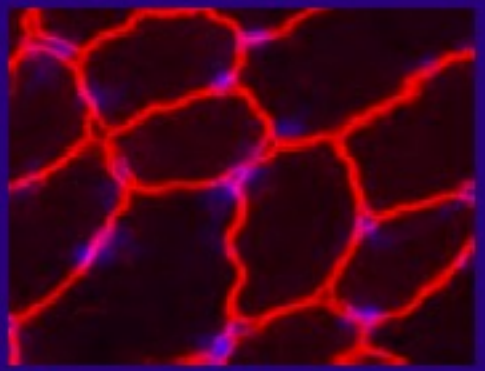

Dystrophin gene - 2.3 Mb
Exons
ATCGGTACGGACT
Introns
S
Cut&past
Splicing
mRNA
R
N-
terminus
cys
C-
terminus
Dystrophic protein


Exons
Mutation
Introns
S
Cut&past
mRNA
R
Stop signal
N-
terminus
cys
C-
erminus
No dystrophic protein

Antisense therapy corrects mutations

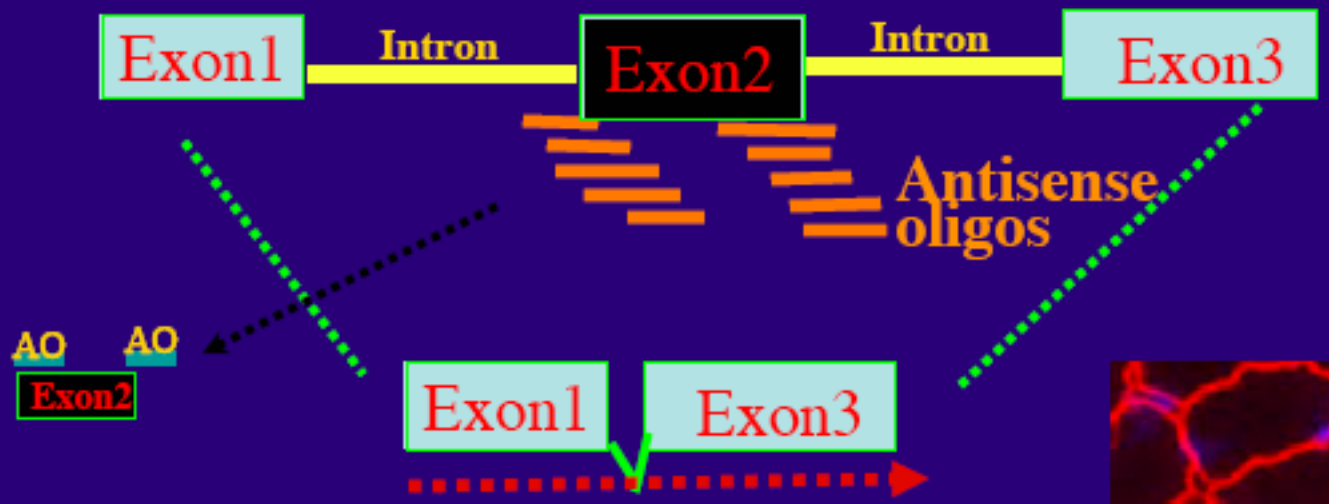


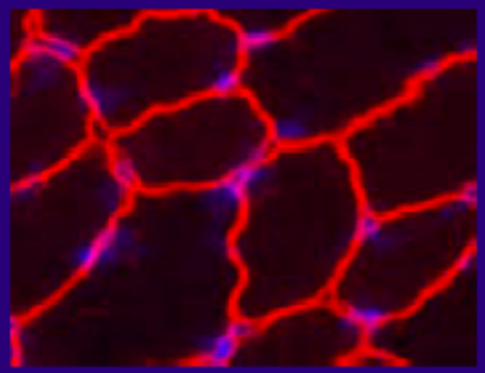




What has been done for the application of antisense therapy for the treatment of DMD?

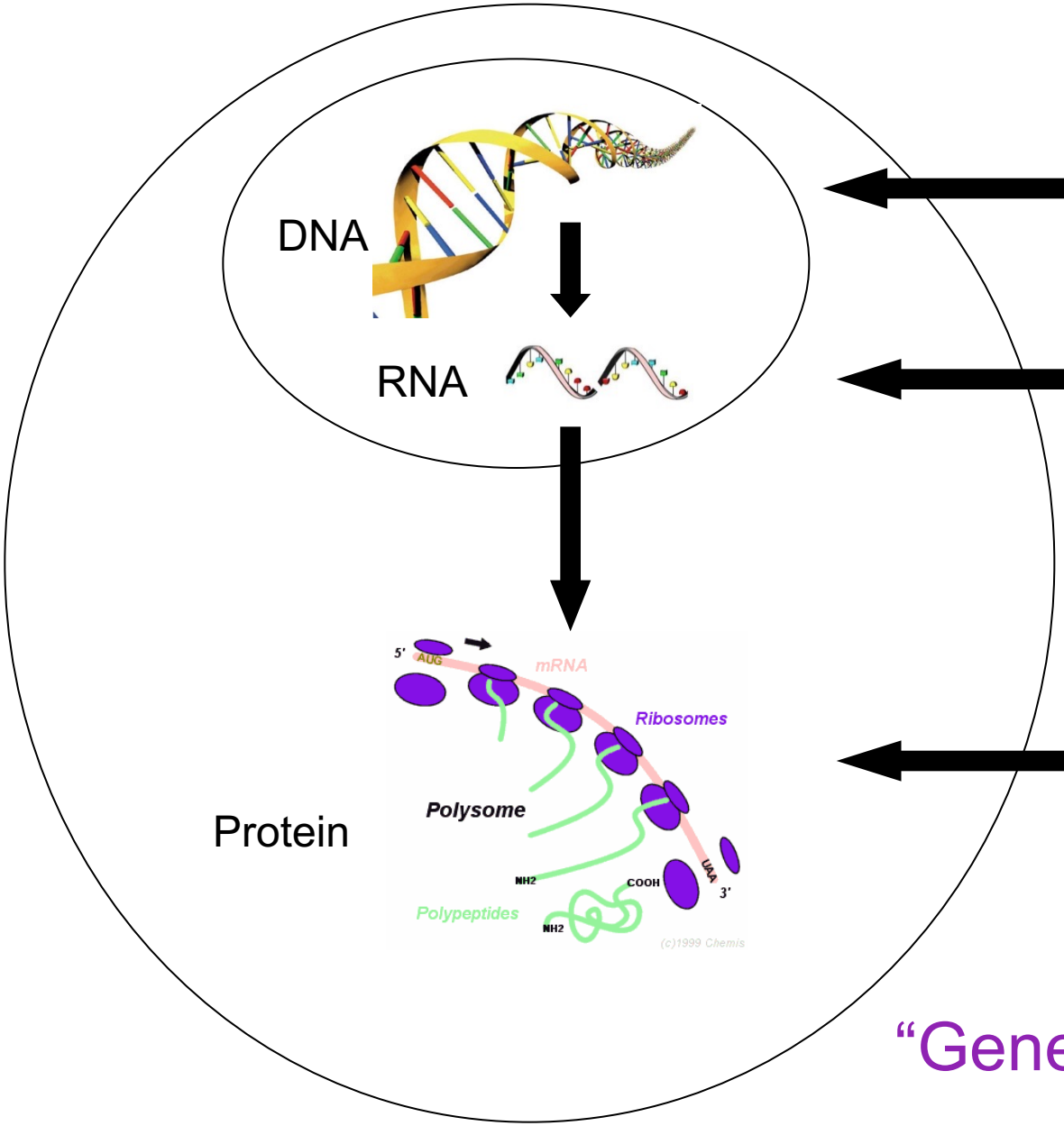
1. Selection of efective antisense sequences





Exon skipping – clinical trials

- Two chemistries taken to clinical trial – 2OmePS and PMO. Evidence of clinical benefit – arresting the progression of the disease for boys eligible for exon 51 skipping.
- Other exon targets current in clinical trial (44, 45, 53).
- Other antisense reagents:
  - Cell penetrating peptides linked to PMO
  - TricycloDNA oligonucleotides



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Cell therapy.

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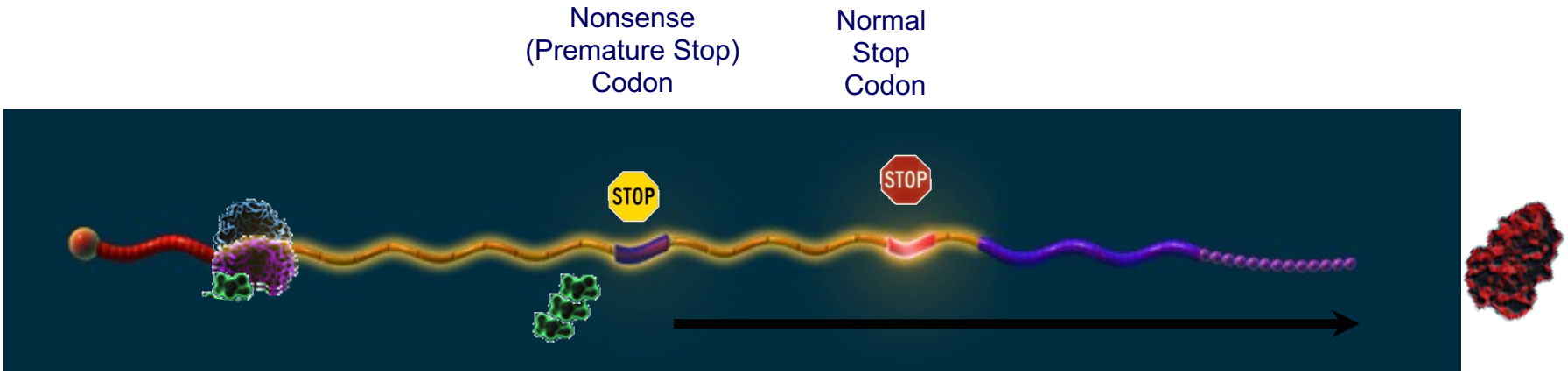
miRNA

Modify translation
e.g. gentamycin
PTC 124 (ataluren)

"Genetic" therapies

~13% of boys have DMD due to a nonsense mutation



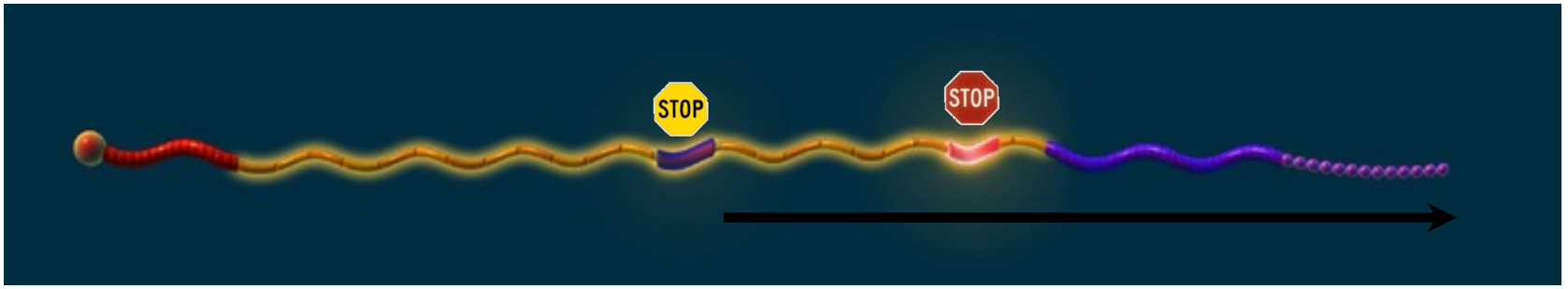
Dystrophin mRNA

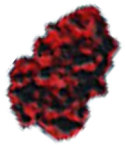
Incomplete dystrophin

Ataluren – ns mutation codon read through

Nonsense
(Premature Stop) Codon

Normal
Stop Codon





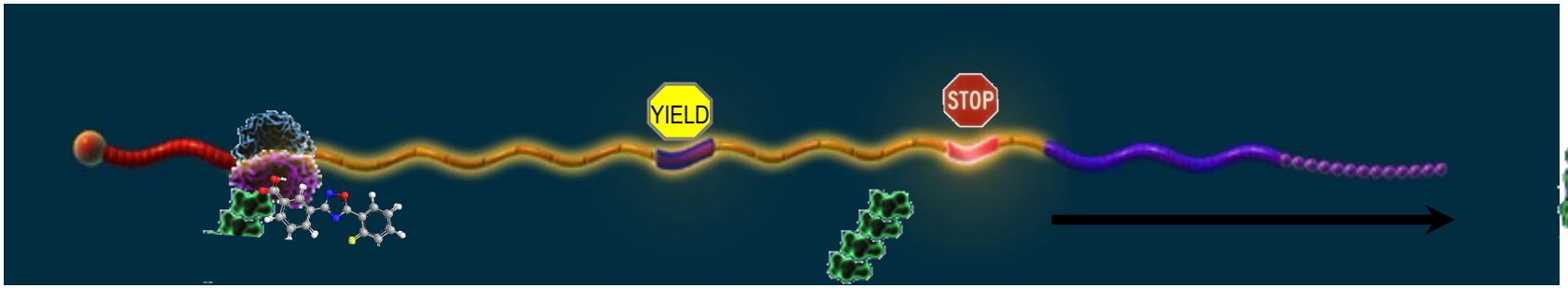
Dystrophin mRNA

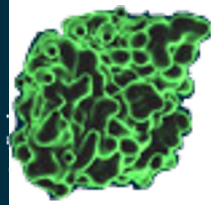
Incomplete
dystrophin

Ataluren induces full-length protein production

Nonsense
(Premature Stop) Codon

Normal
Stop Codon



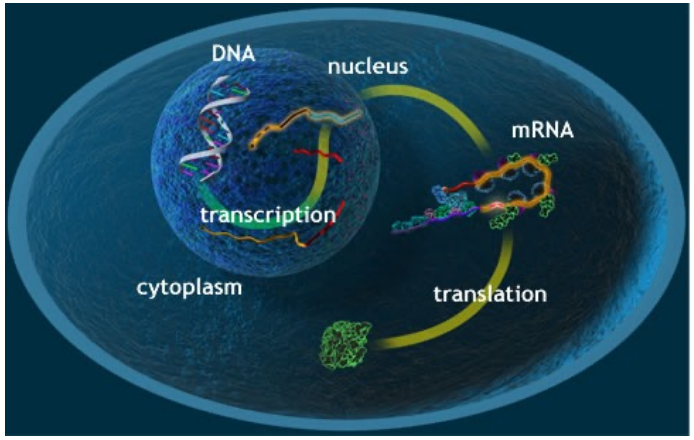


Dystrophin mRNA

Full-length
dystrophin

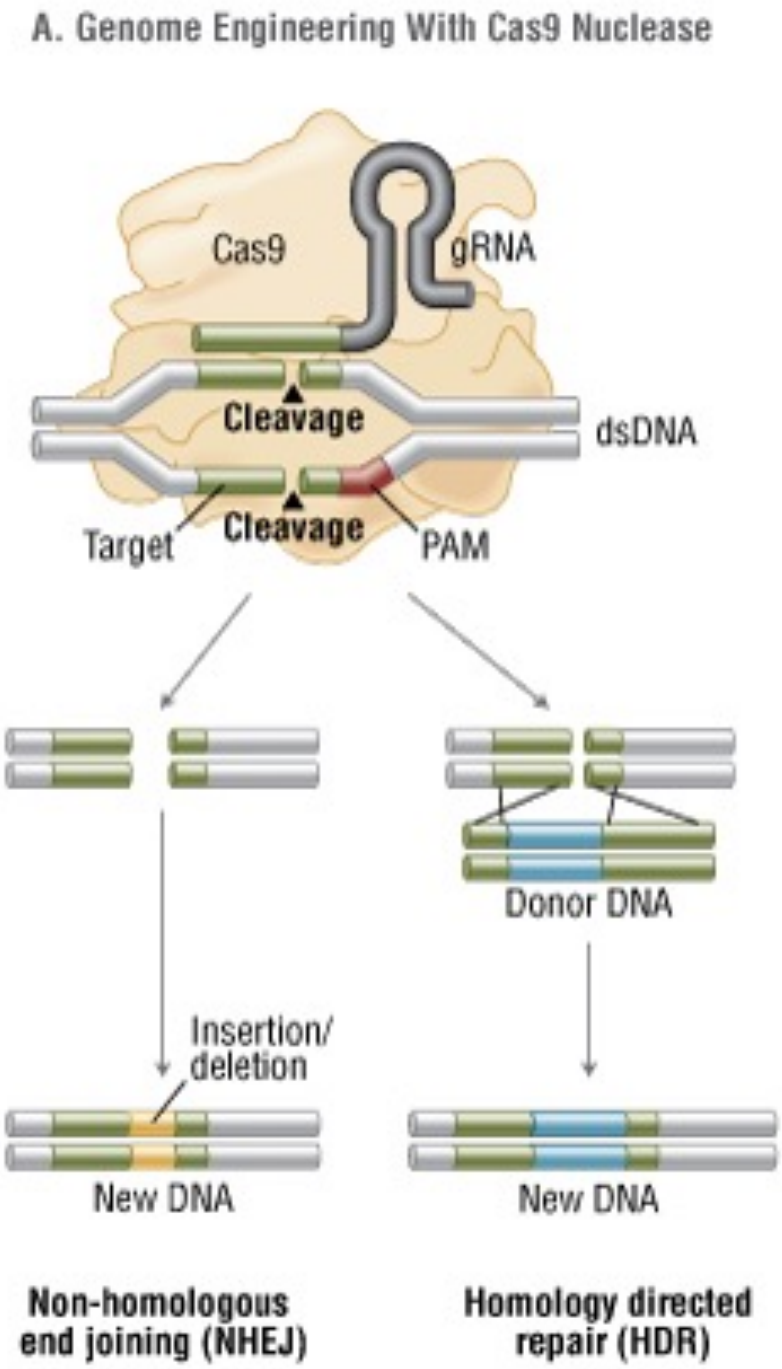
K Bushby ICNMD 2010

Read-through of premature stop mutations



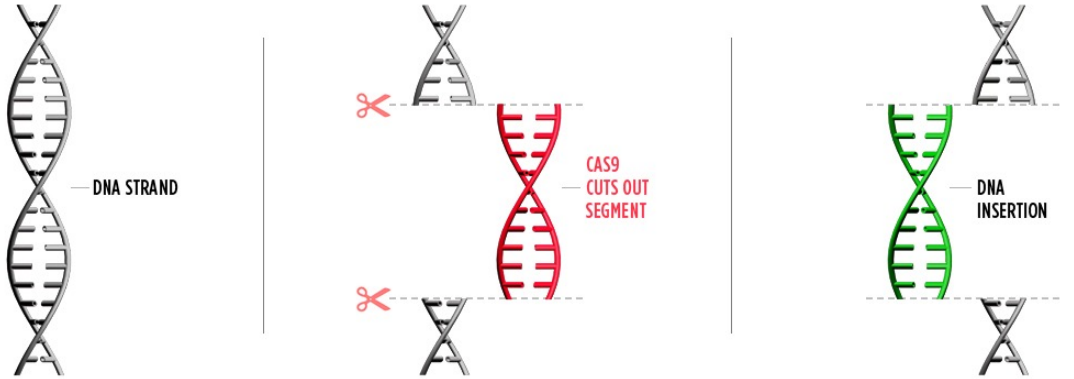
- Approximately 15% of DMD patients have a premature stop mutation.
- Aminoglycoside antibiotics can “read-through” these mutations but are too toxic for long-term human use.
- High-throughput screening identified a much less toxic compound with similar activity (PTC124 – Ataluren).
- EU conditional approval as Translarna for a restricted age range.

PTC THERAPEUTICS


A. Genome Engineering With Cas9 Nuclease
Cas9
gRNA
Cleavage
dsDNA
Target
Cleavage
PAM
Donor DNA
Insertion/
deletion
New DNA
New DNA
Non-homologous
end joining (NHEJ)
Homology directed
repair (HDR)

EDITING HUMAN DNA

A revolutionary new gene-editing technique called CRISPR (Clustered Regularly Interspaced Short Palindromic Repeat) can target and modify DNA with groundbreaking accuracy, changing the way we think about treating diseases.



1 DNA contains all of the information in the human genome, including physical traits as well as mutations.

2 Scientists use the CAS9 enzyme to accurately cut out a specific segment of DNA (*e.g. blindness mutation*).

3 Scientists insert an amended segment of DNA which corrects the mutation (*e.g. vision gene*).

SOURCE: UNIVERSITY OF CALIFORNIA BERKELEY

KQED

GENE EDITING

In vivo gene editing in dystrophic mouse muscle and muscle stem cells

Postnatal genome editing partially restores dystrophin expression in a mouse model of muscular dystrophy

GENE EDITING

In vivo genome editing improves muscle function in a mouse model of Duchenne muscular dystrophy

TERAPIA GENICA EX VIVO DELLA DMD

Limitazioni

- **Cellule bersaglio**
  - disponibilità
  - numero
  - sopravvivenza e proliferazione in vitro
- **Somministrazione**
  - iniezione locale
  - sistemica
- **Trasferimento genico**
  - vettore
  - dimensione del transgene
  - efficienza
  - regolazione dell'espressione genica
  - durata dell'espressione genica
- **Risposta immune contro il prodotto del transgene**

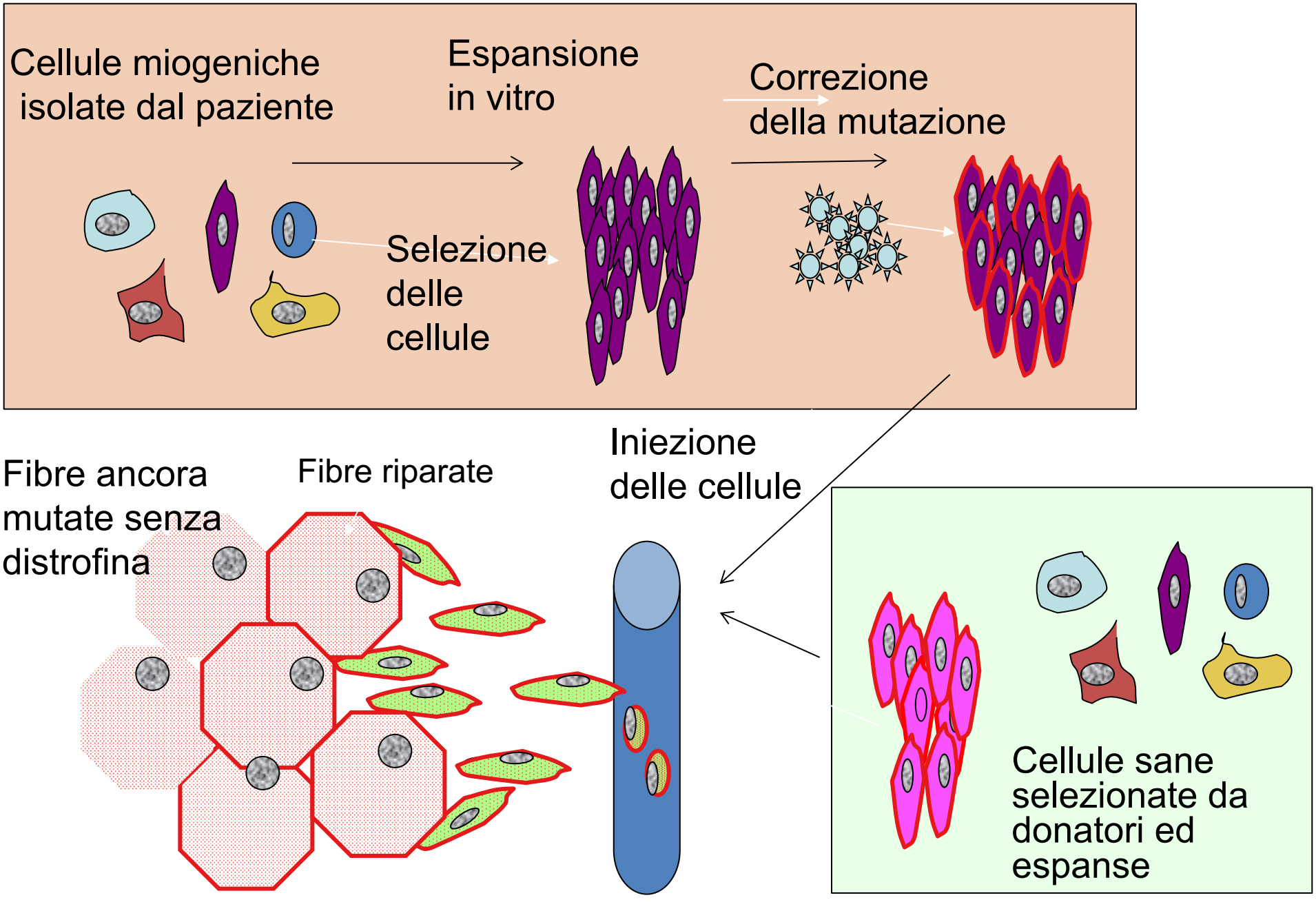
Proprietà ideali delle cellule staminali per la terapia cellulare delle distrofie

- Provenienza: le cellule devono risiedere nel tessuto ed essere facilemente recuperabile mediante biopsia
- Crescita: le cellule devono poter essere mantenute in cultura e crescere in grande numero
- Somministrazione e migrazione : per avere una distribiuzione adeguata di cellule nel muscolo ospite le cellule devono poter essere iniettate in circolo ed essere capaci di extravasare e raggiungere tutti i compartimenti muscolari del corpo.
- Differenziamento: una volta raggiunto il muscolo devono differenziare efficientemente

Possibili strategie per future terapie cellulari delle distrofie muscolari

	Cellule da usare	Vantaggi	Problemi
Eterologo	Cellule staminali da donatore compatibile	Non serve Terapia genica	Immuno-soppressione o tollerizzazione
Autologo	Cellule staminali Isolati dal paziente	Non serve Immuno-soppressione	Uso di vettori virali, Il gene della distrofina è molta grande

Cellule staminali per il riparo delle distrofie



Cellule usate per protocolli sperimentali terapia cellulare della DMD

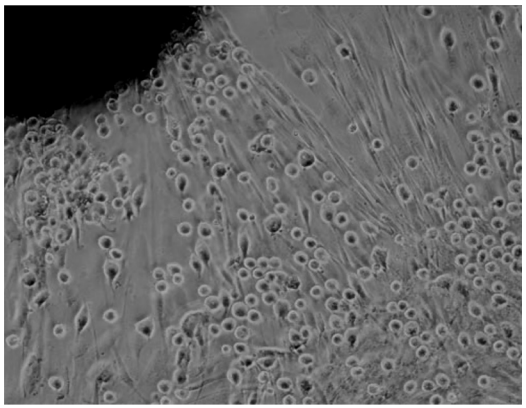
Cellula	Derivazione	Crescita in vitro	Migrazione al muscolo per via sistemica
HSC (SP)	Midollo osseo	limitata	sì
MAPC	Midollo osseo	sì	-
Mesenchymal (MSC)	Midollo osseo	sì	sì
Muscle derived progenitors	Muscolo	sì	modesta
Mesoangioblasti	Piccoli vasi	sì	sì

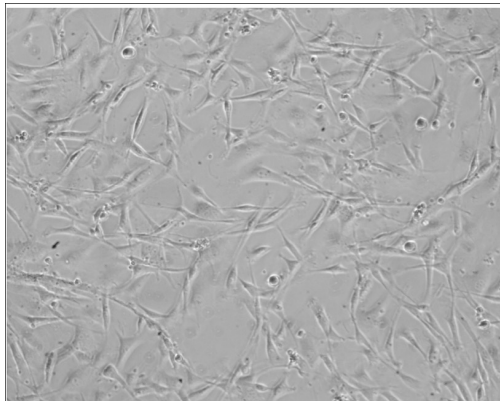
Da piccoli vasi di muscolo adulto di diverse organismo si possono isolare mesoangioblasti che in cultura differenziano in muscolo scheletrico

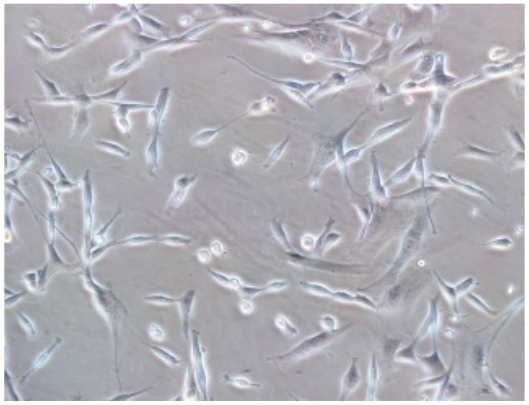
Uomo

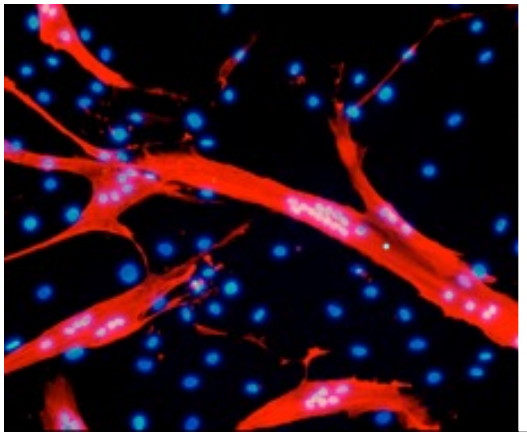
Topo

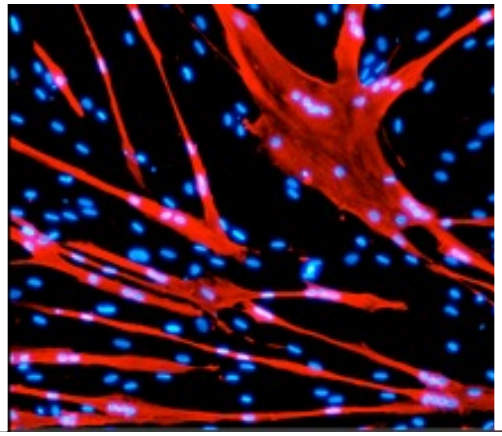
Cane

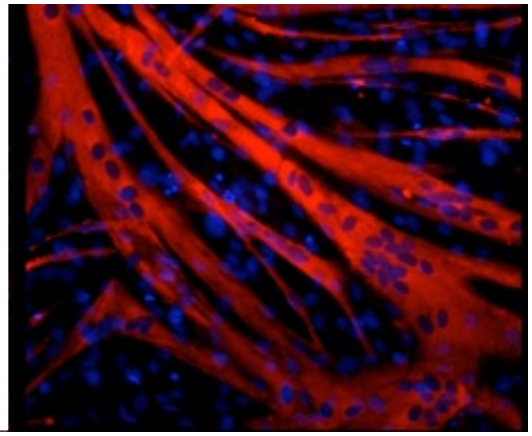




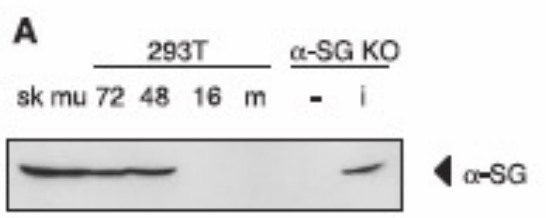


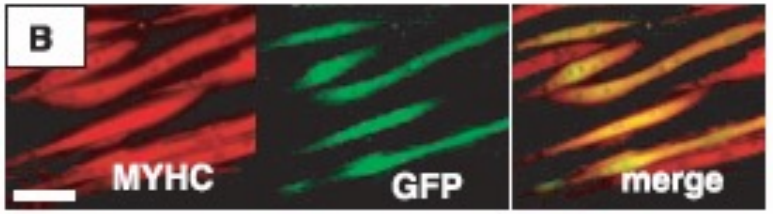


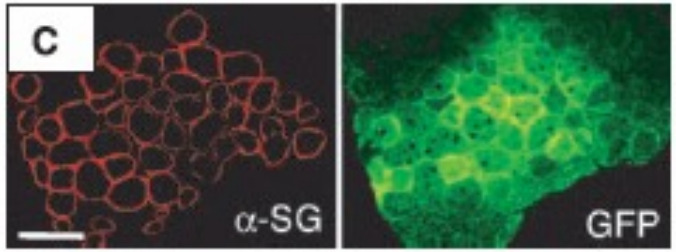


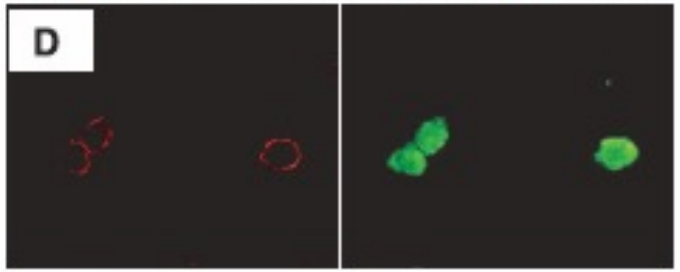


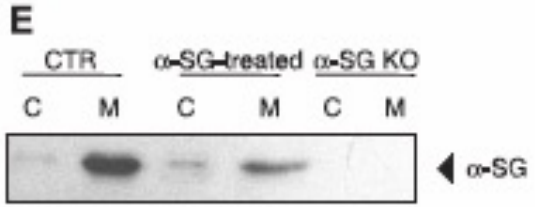
Lentiviral vectors expressing α-sarcoglycan-GFP transduce mutant mesoangioblasts that express the proteins in vitro and in vivo

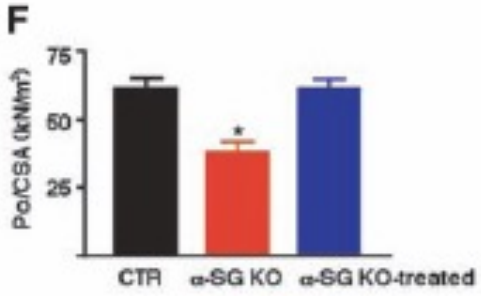




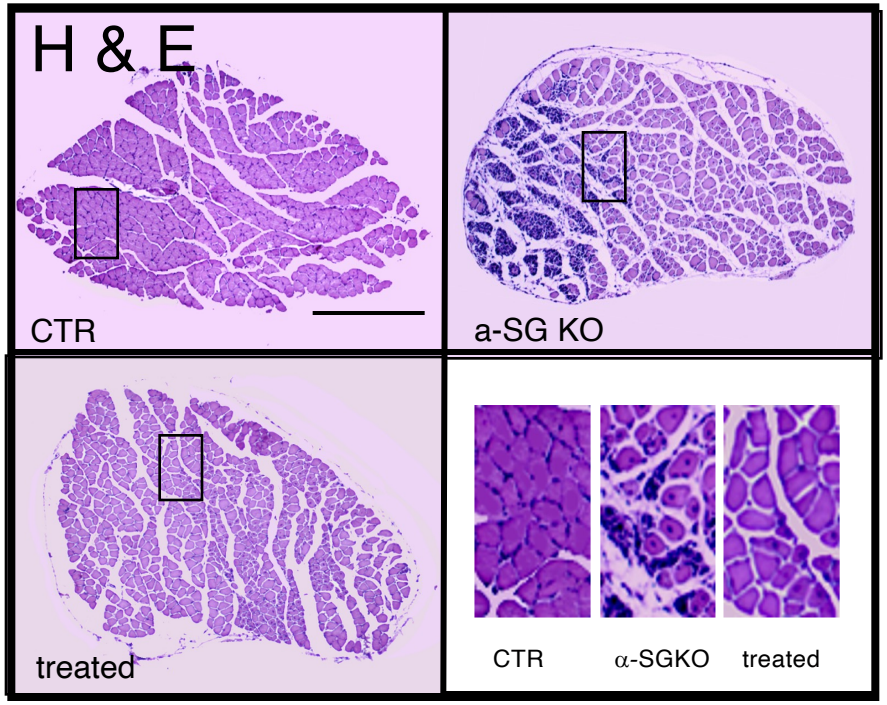


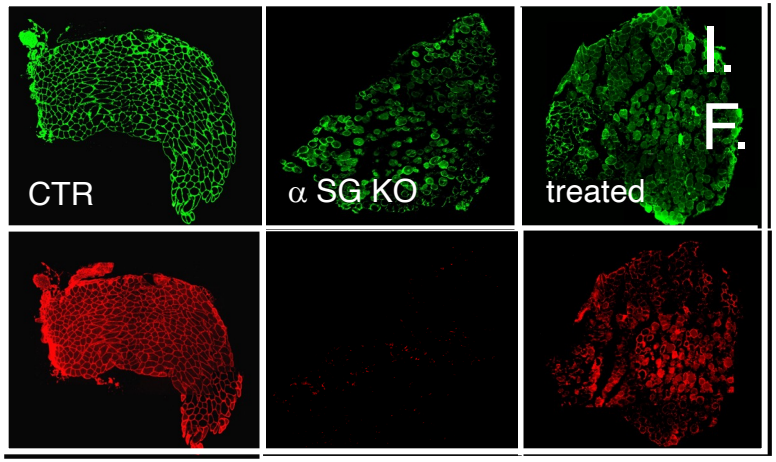


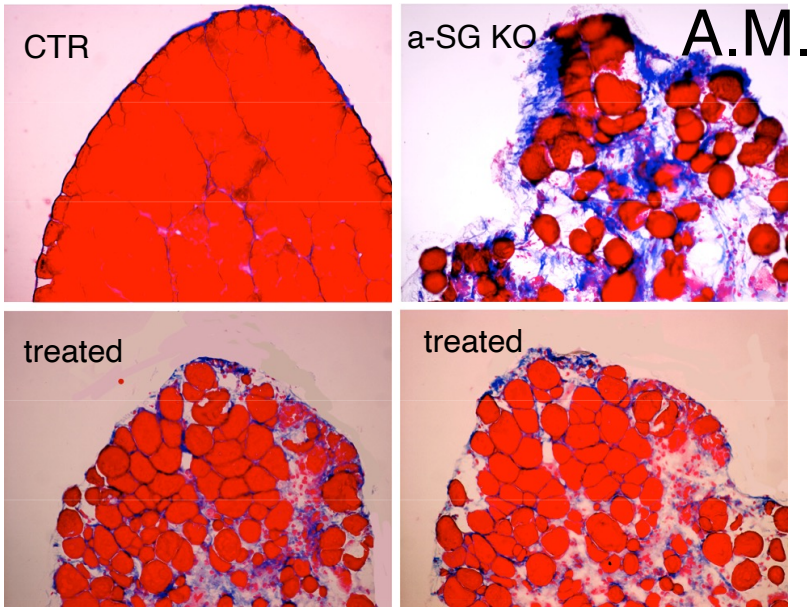




Il trattamento con cellule staminale migliora la morfologia del muscolo dei topi distrofici







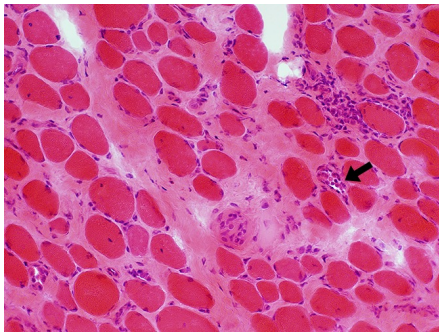
Cell Therapy of α-Sarcoglycan Null Dystrophic Mice Through Intra-Arterial Delivery of Mesoangioblasts

www.sciencemag.org SCIENCE VOL 301 25 JULY 2003

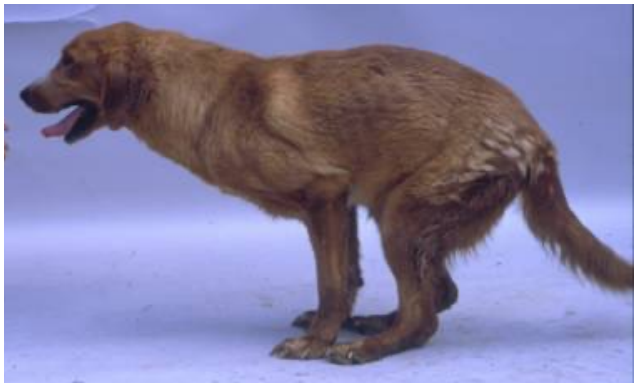
Mutazioni nel gene della DISTROFINA

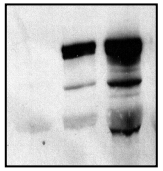
Bambini con DISTROFIA di DUCHENNE
1 su 3500 nati maschi

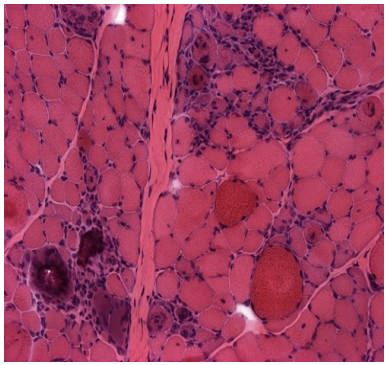
Mediamente l'aspettativa di vita non è superiore ai 20 anni, dai 10 in carrozzina, con ventilazione notturna.



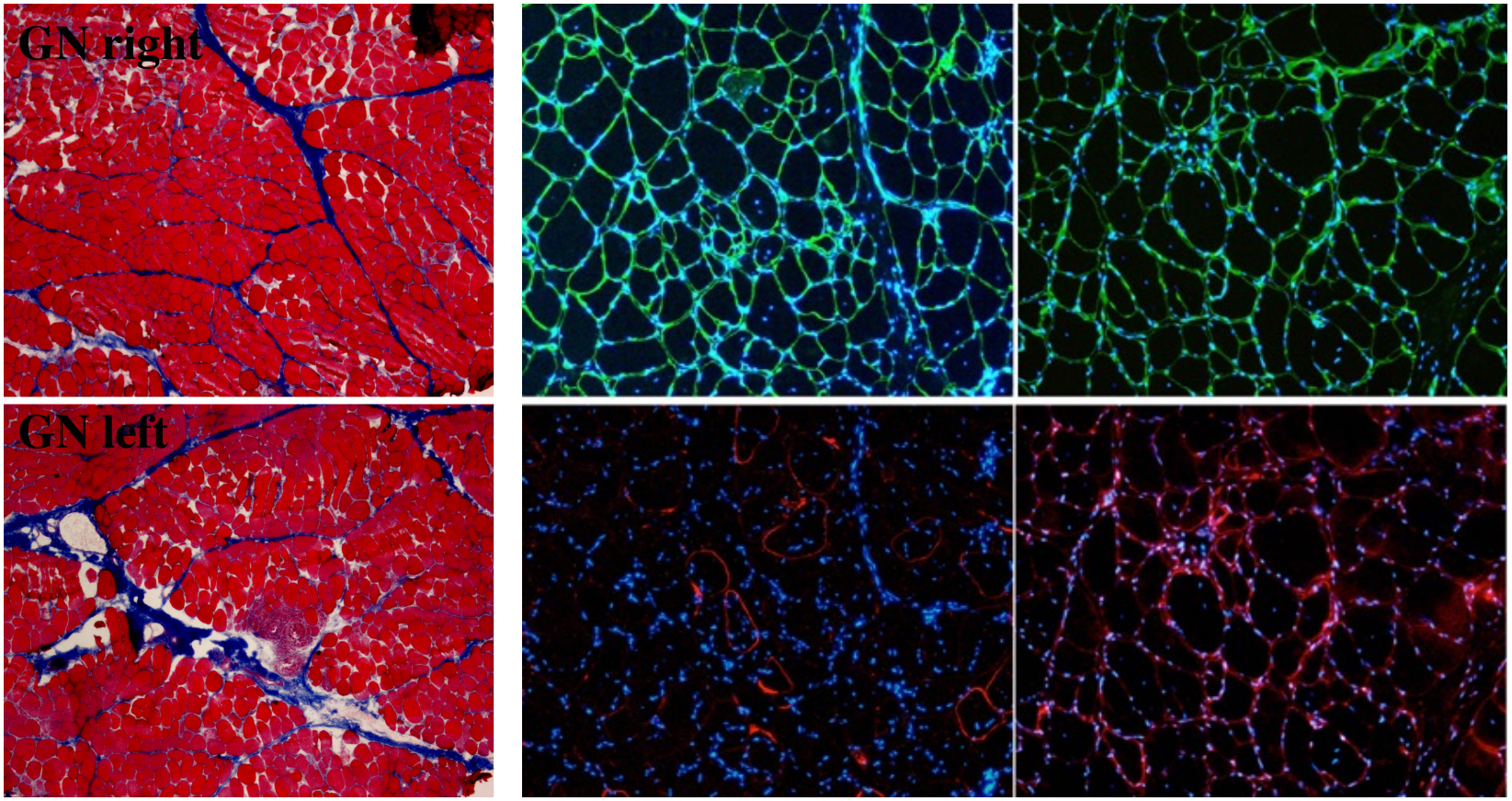
Cane Distrofico GRMD







Anche nel modello canino l'analisi su biopsie muscolari evidenza un miglioramento quando sono somministrate cellule staminali



Laminin = Dystrophin • = nucleo

Mesoangioblast stem cells ameliorate muscle function in dystrophic dogs

A. Protocol Information		
A.1	Member State Concerned	Italy - Italian Medicines Agency
A.2	EudraCT number	2011-000176-33
A.3	Full title of the trial	Cell Therapy Of Duchenne Muscular Dystrophy by intra-arterial delivery of HLA-identical allogeneic mesoangioblasts
A.3.2	Name or abbreviated title of the trial where available	ND
A.4.1	Sponsor's protocol code number	DMD03
A.5.1	ISRCTN (International Standard Randomised Controlled Trial) Number	ND
A.7	Trial is part of a Paediatric Investigation Plan	Information not present in EudraCT
A.8	EMA Decision number of Paediatric Investigation Plan	

https://www.clinicaltrialsregister.eu/ctr-search/trial/2011-000176-33/IT

Published online: November 5, 2015

Research Article

EMBO Molecular Medicine

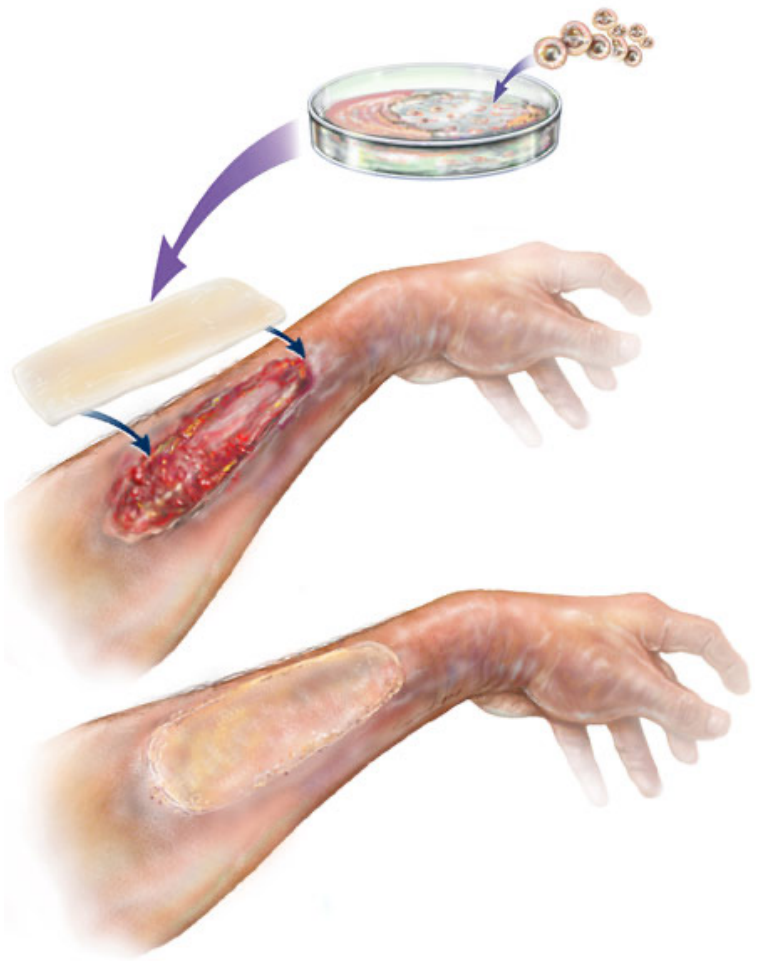
Intra-arterial transplantation of HLA-matched donor mesoangioblasts in Duchenne muscular dystrophy

Conclusioni e prospettive

- Il muscolo contiene diversi tipi di cellule staminali che contribuiscono al suo sviluppo e alla rigenerazione dopo un danno
- Nelle distrofie muscolari il processo di rigenerazione è compromesso
- Diversi protocolli sperimentali su animali modello di distrofia hanno dimostrato che la somministrazione di cellule staminali migliora la morfologia e la funzionalità del muscolo
- Sono in corso trial clinici con cellule staminali in pazienti con Distrofia Muscolare di Duchenne: al momento questi studi hanno dimostrato che il trapianto è una procedura relativamente sicura anche se per arrivare realmente ad una cura è necessaria l'ottimizzazione di diversi parametri quali:
  - Modalità di somministrazione delle cellule (quantità e frequenza)
  - Età di inizio della somministrazione
  - Combinazione con altri farmaci che migliorano il reclutamento delle cellule e il loro differenziamento miogenico
  - Combinazione con terapia genica e uso di nuove tecnologie: gene editing con CRISPR-CAS9 ?

Tissue Engineering

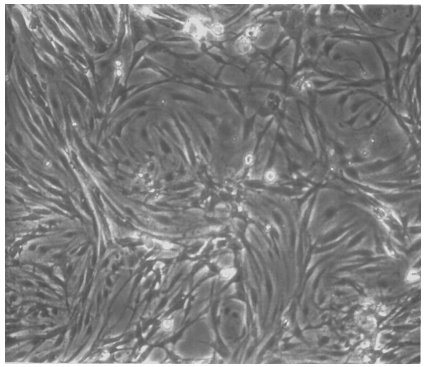
Tissue Engineering is “an interdisciplinary field that applies the principles of engineering and life sciences toward the development of biological substitutes that restore, maintain, or improve tissue function or a whole organ“ (Langer R, Vacanti JP. Science 1993; 260: 920)



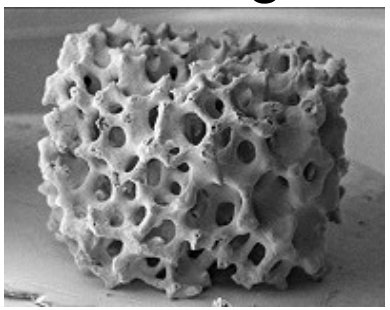
Tissue Engineering approach

biopsy

in vitro cell expansion



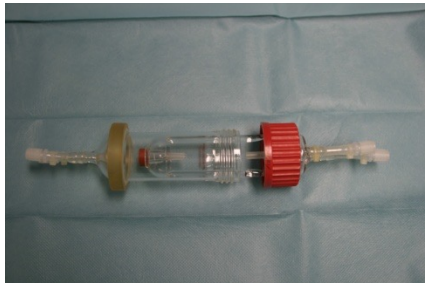
Scaffold seeding



Static culture

Implantation

Dynamic culture



Autologous cells

Primary cells

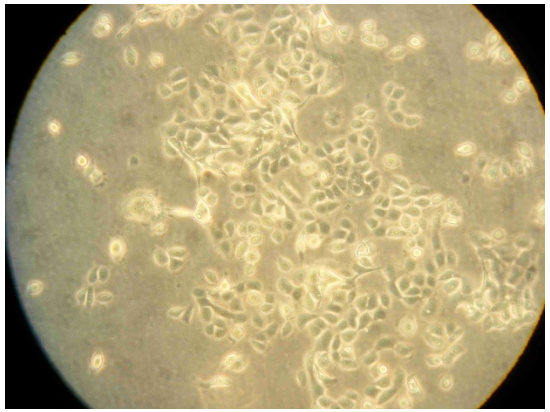
Allogeneic cells

Cell sources

Secondary cells

Xenogenic cells

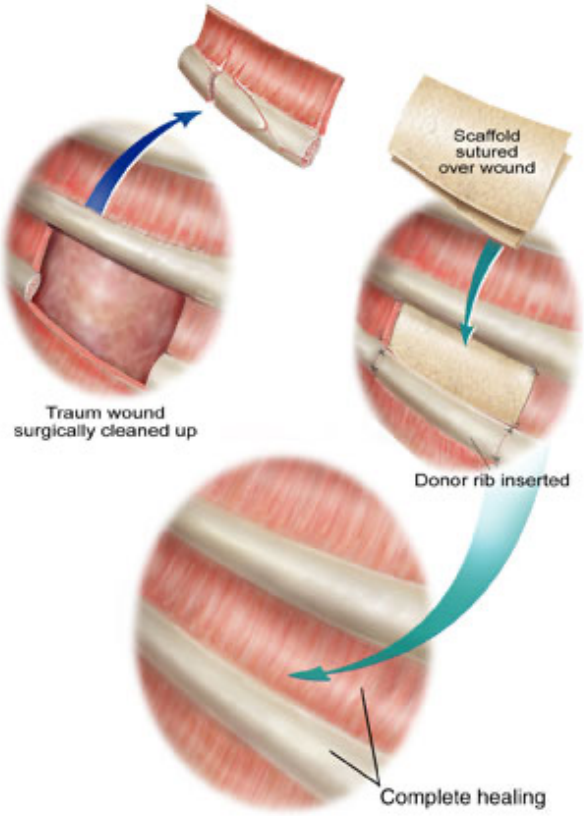
Syngenic or isogenic cells

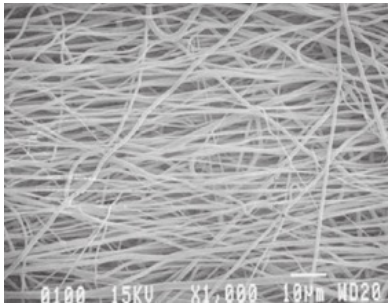


Stem cells

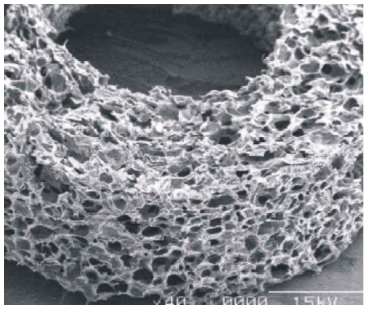
Scaffold

- Allow cell attachment and migration
- Deliver and retain cells and biochemical factors
- Enable diffusion of vital cell nutrients and expressed products
- Exert certain mechanical and biological influences to modify cell behaviour





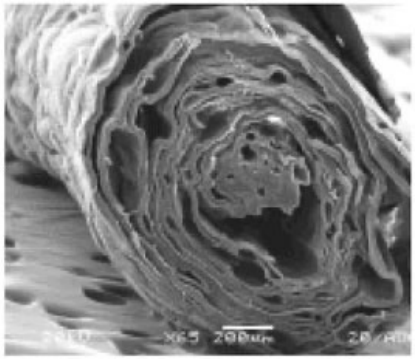
Biomaterials

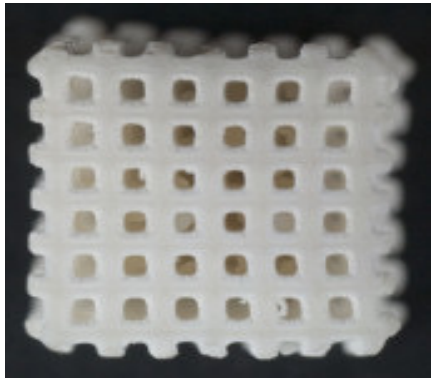


- easy and reproducible manufacture
- biocompatibility
- non-immunogenicity
- suitable resorption rates

Synthetic

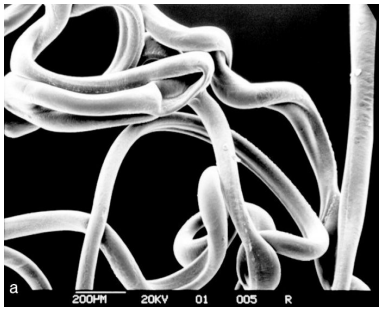
Natural



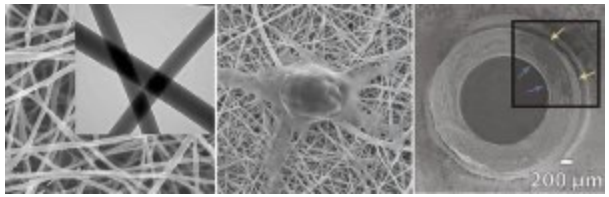


Synthetic biomaterials

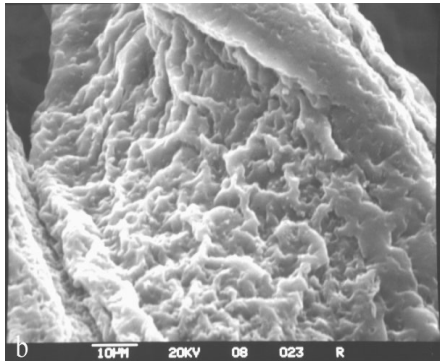
Polylactic acid
(PLA)



Polyglycolic acid
(PGA)



Poly(ε) caprolactone
(PCL)



Natural biomaterials

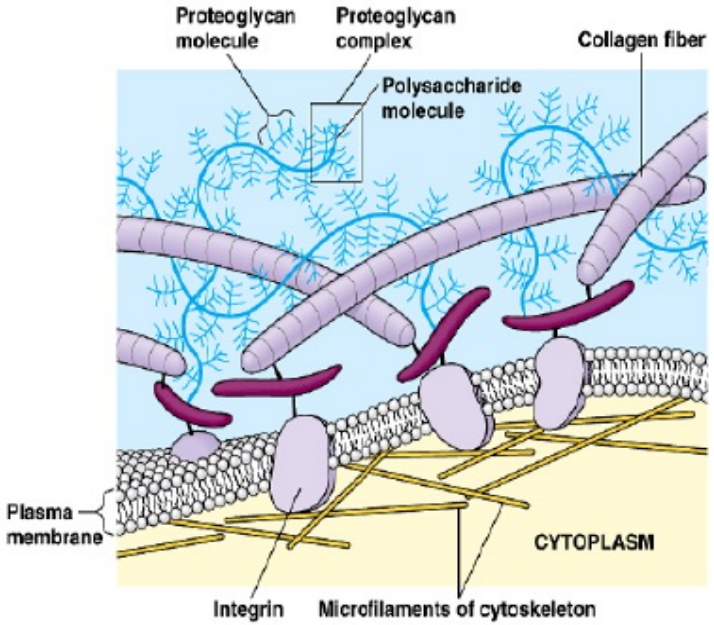
Collagen

Fibrin

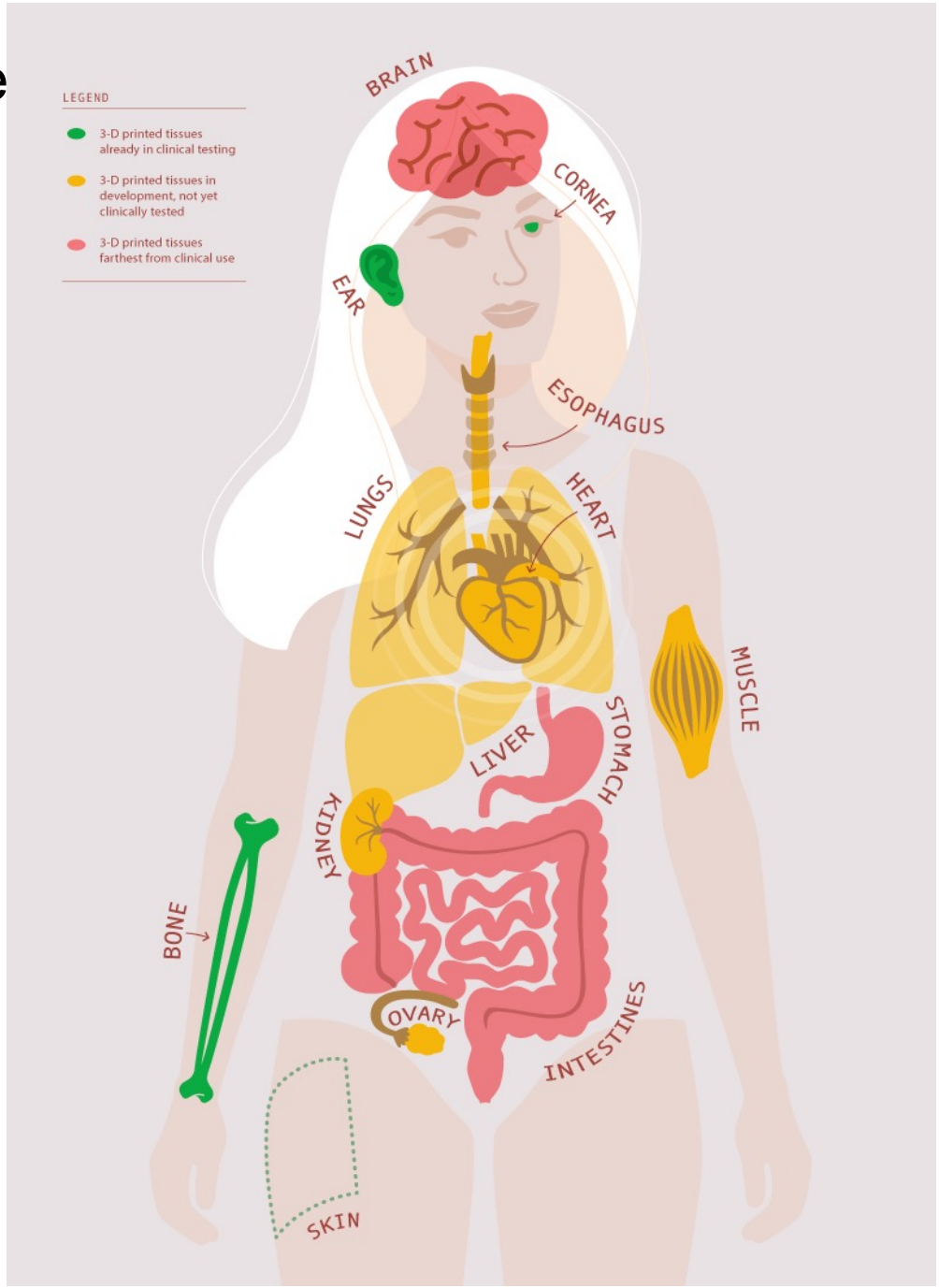
Chitosan

Hyaluronic acid
(glycosaminglycans)

Extracellular matrix



3D printed tissue



Bone – Scientists have 3-D printed scaffolding that is seeded with living tissue that grows into bone. While several preclinical trials are underway, one patient with tibial osteomyelitis, an infection that damaged 36 centimeters of his leg bone, successfully received the transplant in 2017 and is now walking. Clinical trials are planned.

Cornea – A company called Precise Bio reports it has completed initial safety studies in animals of 3-D printed corneas to treat eye injuries, defects, and infections that cause permanent damage. The company has progressed to further preclinical trials in multiple animal models, and expects to begin clinical trial next year.

Ear – Aurinovo received a rare pediatric disease designation for its 3-D printed cartilage designed to treat microtia, a birth defect leading to a misshapen ear, and plans to begin clinical trials this year. Researchers in China have already reported transplanting 3-D printed ears onto children who had birth defects that left

Kidney – Researchers at Organovo 3-D printed a kidney organoid to be used in drug testing.

Liver – Researchers at Organovo are testing patches of liver tissue that could engraft into the liver, rather than transplanting an entire organ.
Lungs – Researchers 3-D printed a vascularized model of an airsac that mimicked the function of lungs.

Muscle – In 2018, Wake Forest researchers published a study in which they implanted patches of 3-D printed muscle tissue into rodents.

Skin – Several groups are working on 3-D printing skin. Researchers from Wake Forest University developed a handheld device to print skin directly onto a patient's wound, which is now entering clinical trials. Last year,